AN INVESTIGATION OF THE INTERACTION BETWEEN MDM2 AND DNA POLYMERASE α

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by

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ABSTRACT

The p53 gene is mutated in more than 50% of human cancers and moreover, alterations in p53 regulated/regulatory pathways are present in the vast majority of cancers. Taking into account the number of cellular processes and pathways that p53 is part of, it is not surprising that loss of p53 function has an influence on many aspects of carcinogenesis. In addition to p53 inactivation through mutations, a well documented process leading to abrogation of p53 functions and stability is over-expression of MDM2 – essential negative regulator of p53. A growing body of evidence indicates that MDM2 can also promote cancer in a p53-independent manner. The oncogenic potential of MDM2 appears to be mainly the result of protein-protein interaction. One such MDM2 interacting protein that was previously identified in our laboratory is DNA polymerase ε. Serendipitously, during the course of MDM2-DNA polymerase ε studies, it was also discovered that MDM2 interacts with DNA polymerase α (these two protein complexes co-purify in partially overlapping fractions from mammalian cells). The main aim of the work presented in this thesis was to further investigate the MDM2 interaction with DNA polymerase α by performing experiments both *in vitro* and in cells in culture. The results of *in vitro* studies indicate that MDM2 can interact directly with the catalytic subunit of DNA polymerase α (p180) and that first 166 amino acids of MDM2 protein seem to be required for this interaction. Moreover both the carboxy- and amino- termini of p180 contribute to this interaction. In addition, MDM2 appears to be capable of stimulating the DNA polymerase activity of DNA polymerase α *in vitro*. We have also demonstrated that p180 can be modified by ubiquitylation in the cells although surprisingly, the role (if any) of MDM2 in this event, may be to suppress this modification. Finally, and perhaps most revealingly, MDM2 preferentially forms a complex in cells with a non-/hypophosphorylated form of DNA polymerase α which has been shown to be involved in initiation of DNA replication, thus providing a possible link between MDM2 oncogenicity and S-phase progression. In conclusion, the studies presented in this thesis describe direct and indirect interaction of MDM2 with DNA polymerase α, a key enzyme involved in the initiation of DNA replication and in so doing may shed light on mechanism/s through which MDM2 might mediate some of its p53-independent oncogenic effects.

Keywords: DNA polymerase α, MDM2, p53

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DECLARATION OF ORIGINALITY

This thesis is a result of my own work performed during the course of studies in the Division of Surgery and Oncology, University of Liverpool, between January 2006 and February 2009. All the experiments were performed by me with the exception of column affinity chromatography presented in Figure 3.1.1, the experiment was performed by Dr. M. Maguire and Dr. P. Nield; immunoprecipitation experiment presented in Figure 3.2.1 was performed by Dr. M. Maguire and DNA polymerase assay presented in Figure 3.3.17 was performed by Prof. S. Linn at U.C. Berkley. The thesis was written wholly by me under guidance of my supervisors Dr. N. Vlatković and Dr. M. Boyd.

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LIST OF ABBREVIATIONS

A	Aprotinin
aa	Amino acids
Ab	Antibody
APS	Ammonium persulphate
ARF	Alternative reading frame
ATM	Ataxia telangiectasia mutated protein kinase
ATP	Adenosine triphosphate
BAI1	Brain-specific angiogenesis inhibitor
BAX	Bcl-2 associated X protein
BIA	Biomolecular interaction analysis
BPB	Bromophenol Blue
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
Cdc6	Cell division cycle 6
Cdc45	Cell division cycle protein 45
CDK	Cyclin dependent kinase
Cdt1	Chromatin licensing/DNA replication factor 1
Chk	Check point kinase
Co-IP	Co-immunoprecipitation
Daxx	Death-domain-associated protein
DHFR	Dihydrofolate reductase
DMs	Double minutes
DMSO	Dimethyl sulfoxide
DNA	Deoxy-ribonucleic acid
DNA-PK	DNA-activated protein kinase
dNTPs	Deoxynucleotide triphosphates
DTT	DL dithiothreitol
ECL	Enhanced chemiluminescence
E.coli	Escherichia coli
EDTA	Ethylene diamine tetracetic acid

FBS	Fetal bovine serum
GADD45	Growth arrest and DNA damage-inducible gene 45
GINS	Go, Ichi, Nii, San-Japanese; five, one, two, three-English
GML	Glycosylphosphatidylinositol-anchored molecule-like protein gene
GST	Glutathione S-transferase
HA	Hemagglutinin
HAUSP	Herpes-associated ubiquitin-specific protease
HECT	Homologous to E6-associated protein C terminus
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HRP	Horseradish peroxidase
IB	Immunoblot
IP	Immunoprecipitation
IPTG	Isopropyl – B – D –thiogalactopyranoside
kDa	kilo Daltons
L	Leupeptin
LB	Luria Bertani medium
MCM	Minichromosome maintenance
MDM2	Murine Double Minute-2
MOI	Multiplicity of infection
MTBP	MDM2 (two) binding protein
Nbs1	Nijmegen breakage syndrome 1
NEDD8	Neural precursor cell expressed, developmentally down-regulated 8
NES	Nuclear export signal
Ni-NTA	Nickel-nitrilotriacetic acid
NLS	Nuclear localization signal
NoLS	Nucleolar localization signal
ORC	Origin recognition complex
ORF	Open reading frame
P	Pepstatin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIs	Protease inhibitors

PI3-K	Phosphatidyl inositol 3-kinase
PMSF	Phenylmethylsulphonyl fluoride
pre-IC	Pre-initiation complex
pre-RC	Pre-replicative complex
Rb	Retinoblastoma protein
RING	Really interesting new gene
RNA	Ribonucleic acid
RPA	Replication protein A
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
Ser	Serine
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
STI	Soybean trypsin inhibitor
SUMO	Small ubiquitin-like modifier
SV40	Simian virus 40
TEMED	N,N, N',N' – tetramethylethylenediamine
Thr	Threonine
TSL	Translesion synthesis polymerases
TSP1	Thrombospondin 1
uORFs	Upstream open reading frames
UV	Ultraviolet
VHL	Von Hippel – Lindau

CHAPTER I

INTRODUCTION

1.1 Background on cancer

Cancer can be described as a group of diseases characterized by uncontrolled cell growth and the ability of such cells to invade adjacent tissues and spread throughout the organism. This type of cell behavior is caused by alterations in the cell genome resulting in transformation of normal cells into malignant cancer cells. Alterations in the genome can occur as an effect of either exogenous factors such as ionizing radiation, UV radiation or mistakes introduced into the genome during endogenous cellular processes such as DNA synthesis. Generally speaking, there are two categories of genes whose alterations contribute substantially to the development of cancer. One category comprise genes mainly involved in promoting cell growth and differentiation whose alteration leads to their activation, whilst the second category includes genes that are inactivated when mutated, resulting in disruption of cell cycle control or apoptosis promotion. The latter group of genes are called tumour suppressors, while the former proto-oncogenes (known as oncogenes when altered in cancer). When oncogenic mutations occur in oncogenes they are dominant, which means that only one defective copy of the gene is enough to contribute to carcinogenesis, whereas in general, mutations in tumour suppressors are considered to be recessive; therefore two copies of a gene need to be mutated in order for altered function of the gene to be manifested (17).

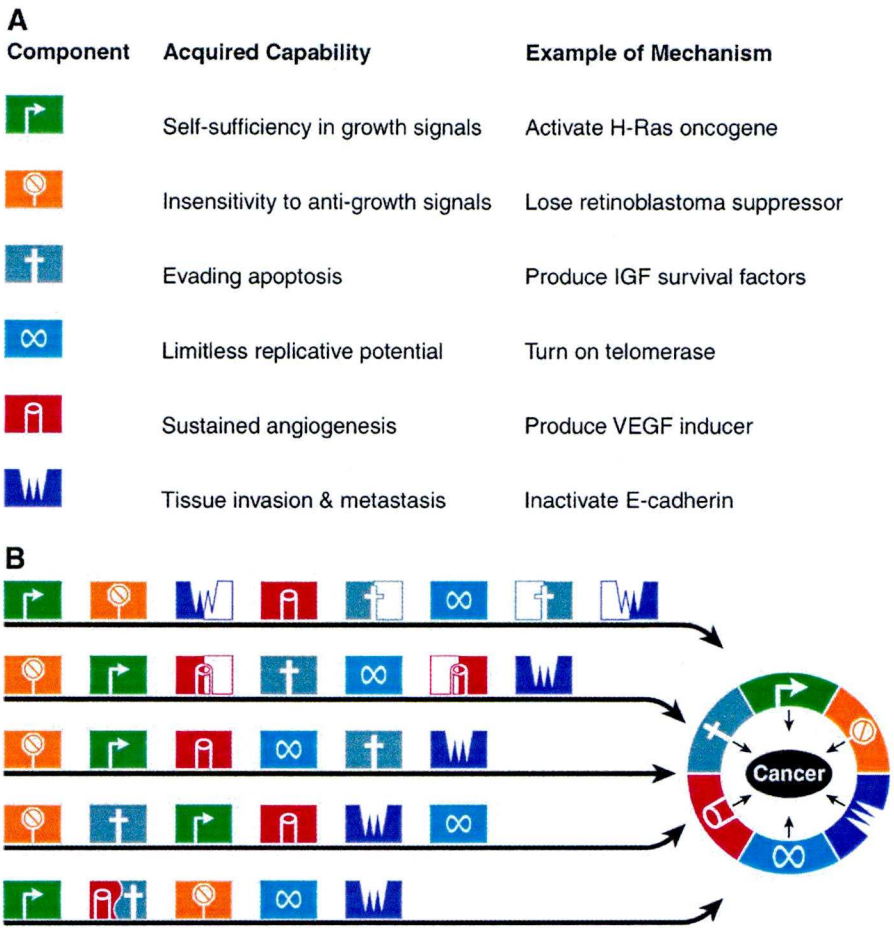


Figure 1.1.1 Hallmarks of cancer. Characteristic of cancer cell with (A) examples of mechanism involved and (B) proposed "order of appearance" in the cell (figure reproduced from reference 74).

Acquired genomic alterations can have substantial consequences on many disparate functions of the cell, but only certain combinations can lead to cancer development. These critical "consequences" have been organised by Hanahan and Weinberg into six phenotypic traits by which cancerous cells can be characterized and distinguished from normal ones. These "hallmarks of cancer" include: self sufficiency in growth signals, insensitivity to growth - inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, tissue invasion and

metastasis. These properties, which can be found in all types of human cancers, are acquired gradually and the "order of appearance" may vary in different types of cancer or even within a type of cancer as presented in Figure 1.1.1 (74).

Carcinogenesis is typically a long, multistep process and since genome alterations accumulate with time, cancer is more likely to develop in old age. However, the occurrence of mutations in germline cells allow for their transfer from generation to generation. Thereby some people harbour hereditary predispositions to cancer syndromes such as: Li Fraumeni (mutations in p53 gene) and Von Hippel – Lindau (mutations in VHL gene) that increase the probability of affected individuals developing cancer early in a lifetime (17,191,192). Most cancers are however sporadic and result from somatic mutations which are not inherited.

1.2 An introduction to MDM2

Among all of the genes found in an altered form in cancer cells, one deserves special attention - the p53 gene, which has been observed to be mutated in approximately 50% of all human cancers (187). p53 is a transcription factor for a number of genes involved in key processes in cells such as cell cycle control, apoptosis, DNA repair, angiogenesis and motility. Therefore, it is not surprising that inhibition of p53 function affects almost every aspect of cancer development (193). In addition to mutations the activity of p53 can be abrogated as a result of negative regulation by some oncogenes, with the most notable one being MDM2. MDM2 exerts its inhibitory activity on p53 by inhibiting its role in transactivation, and furthermore by mediating its ubiquitylation and subsequent degradation by proteasomes. For this reason, MDM2 is therefore an important and widely studied

protein participating in cancer development. MDM2 can also promote cancer in a p53-independent manner as will be discussed later in this chapter (2,18).

The first report on murine double minute 2 (Mdm2) was published more than twenty years ago, wherein *Mdm2* was identified as one of three genes amplified on small chromatin bodies known as double minutes (DMs) in a spontaneously transformed derivative of the murine 3T3 cell line (3T3DM) (4). Further research revealed that *Mdm2* was the only one of these three genes whose overexpression enhanced the tumorigenic potential of NIH3T3 and Rat3 cells (3). Since then up-regulation and overexpression of *MDM2* has been reported in many human tumours (140).

The oncogenic properties of MDM2 are displayed when the *MDM2* gene, located on chromosome 12 (in humans), is amplified or overexpressed in a cell (140, 141). The *MDM2* gene consists of 12 exons and its expression is under the control of two promoters: P1, located upstream of exon 1, and P2 which lies in intron 1 (142,143,144). P1 is constitutively expressed, whereas P2 has two p53 response elements and is regulated by p53 (143,144,145). Transcripts from both promoters encode full length MDM2 protein (p90), with the first start codon of the open reading frame (ORF) being located in exon 3, however there is a difference in the 5'UTR which has a significant impact on translation of the mRNAs originating from the two promoters (143). The 5' untranslated region (5'UTR) is longer in transcripts from the P1 promoter (long, L-MDM2) compared to the 5' UTR expressed from the P2 promoter (short, S-MDM2) transcripts (146). Moreover, L-MDM2 contains two upstream open reading frames (uORFs) which affect its translational potential by reducing ribosome loading and as a result L-MDM2 is about ten times less efficiently

translated than S-MDM2 (147).

In addition to alternative translation the level of MDM2 mRNA varies among individuals as a result of a single nucleotide polymorphism (SNP), either T or G at position 309 located in the P2 promoter region which can cause increased MDM2 levels, due to greater DNA binding affinity of transcriptional activator Sp1 for the G form of the SNP (148).

Apart from the full length p90 protein, many shorter versions of MDM2 are produced and these products are present in tumours as well as in normal tissues. Since 1996, when first alternatively spliced *MDM2* transcripts were described, around forty different MDM2 splice variants have been identified (reviewed in 20, 21). The mechanisms responsible for generation of these variants are usually either alternative or aberrant splicing resulting in loss of complete exons or fragmented exons, respectively. Many variants lack either part of, or the entire p53 binding domain, which has been argued as further evidence for non-p53 regulatory functions for these proteins. In fact, several MDM2 splice variants that are unable to bind to p53 have been shown to promote tumourigenesis, supporting the notion that MDM2 has p53-independent oncogenic potential (36, 93). However it is hard to determine whether proteins are being generated from all the variants and the functions of most alternatively spliced transcripts remain unclear.

1.2.1 Protein domains of MDM2

The MDM2 full length protein consists of 489 amino acids (aa) in mice/491 aa in humans. There are several different domains and motifs that have

been described within MDM2 according to their function and/or structure and these are shown in Figure 1.2.1. The first conserved domain consisting of approximately 100 aa - is located at the very amino terminal end of MDM2 and is called the p53 binding domain thus reflecting the role it plays within the protein. MDM2 binds to the transactivation domain of p53 via a hydrophobic binding pocket formed within residues 26-108 of MDM2 and this results in an inhibition of p53 transcriptional activity (179).

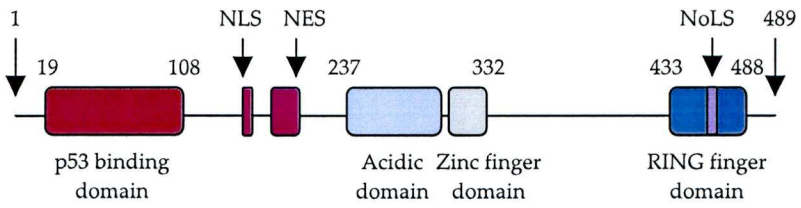


Figure 1.2.1 Structure of full length murine MDM2. The protein consists of 489 amino acids (based on information provided by reference 2). Location of different domains and motifs indicated by coloured boxes; NLS – nuclear localization signal, NES – nuclear export signal, NoLS – nucleolar localization signal.

The nuclear localization signal (NLS) and nuclear export signal (NES) motifs are localized between amino acids 170 - 200 and are responsible for MDM2 shuttling between the cytoplasm and the nucleus. It seems that MDM2 shuttling is related to the function it performs in the cell as a negative regulator of p53, because interaction between both proteins in the nucleus leads to inhibition of p53 transactivation while export to the cytoplasm allows for ubiquitylation and degradation of p53 to occur due to MDM2 E3 ligase activity (2,180).

The central portion of MDM2 contains an acidic domain located between aa 237 – 300 which is involved in binding of a number of MDM2-interacting proteins including ARF, MTBP and ribosomal protein L5 (151). Several reports suggest that the acidic domain plays an important role in MDM2 mediated ubiquitylation and thus influences subsequent degradation of p53. Deletion of the acidic domain or binding of ARF to this region have been demonstrated to result in inhibition of MDM2 – mediated ubiquitylation of p53 (61,181,182).

There is a zinc finger localized next to the acidic domain, whilst a RING finger domain, approximately 60 aa long, occupies the carboxyl terminal end of the protein. The RING finger domain, which also contains a nucleolar localization signal (NoLS) between aa 464 – 471, has been shown to be responsible for MDM2-MDM2 or MDM2-MDMX protein assembly and also for the E3 ligase ubiquitin activity of MDM2 (19).

1.2.2 E3 ubiquitin ligase activity of MDM2

MDM2 is a RING E3 ubiquitin ligase, this means that it can promote the ubiquitylation process, which entails covalent attachment of one or multiple ubiquitin moieties to the substrate protein and in so doing may decide its fate (64, 149). This multi-step process begins with the activation of ubiquitin by an E1 activation enzyme in cooperation with ATP, followed by ubiquitin transfer to an E2 ubiquitin conjugating enzyme. The next step, ubiquitin attachment to the substrate, slightly differs depending on the type of E3 ligase engaged in this process. RING (really interesting new gene) type E3 ligases, such as MDM2, promote direct ubiquitin transfer to a substrate protein, whereas E3 ligases with the HECT (homologous to

E6-associated protein C terminus) domain first transfer ubiquitin to the HECT domain and then to the substrate. As a result of ubiquitylation, the carboxy-terminal end of ubiquitin (actually the absolute carboxy-terminal glycine residue) is covalently linked to lysine residues of target proteins (149). Monoubiquitylation usually leads to a change in localization or activity of target proteins, whereas polyubiquitylation (attachment of at least four ubiquitins moieties) usually results in proteosome dependent degradation of the substrate protein (149,183). MDM2 is capable of mediating both: mono- and polyubiquitylation via its RING finger domain and it has been demonstrated that any disruption in the RING finger domain located on C-terminal end of the protein affects the E3 ligase ubiquitin activity of MDM2 (64,151). A RING domain is generally characterized by the presence of multiple conserved cysteines (usually with one [C3HC4] or a couple [C3H2C3] of histidines) forming a structure capable of binding two zinc atoms (184). Mutational studies performed on the RING domain of MDM2 (C2H2C4) have shown that deletion of the RING domain or substitution of any cysteine residue results in abrogation of zinc binding, causing loss of E3 ubiquitin ligase activity (64,84,184). Moreover, in order to perform the function of an E3 ligase, it is necessary for MDM2 to form either a homodimer or a heterodimer with the homologous protein MDMX and this also depends on the RING domain (150).

MDM2, as an E3 ligase, has many specific substrates (151) with probably the most important one being tumor suppressor p53 (81) (this relationship will be discussed later in more details). In addition to promoting ubiquitylation of other proteins, MDM2 can also mediate its own ubiquitylation leading to proteosomal degradation (64). The ability of MDM2 to promote ubiquitylation and degradation of

p53 and itself can be affected by modifications of MDM2 and also by interactions with different proteins, especially under stress conditions (100). One such example is MDM2 modification by the small ubiquitin-like modifier 1 (SUMO-1) mediated by Ubc9 (E2 SUMO conjugating enzyme) (88). It seems that sumoylation of MDM2 prevents its self-degradation, while p53 ubiquitylation mediated by MDM2 is enhanced. This process may be regulated partly by SUSP4, a SUMO-specific protease whose expression is induced upon UV damage, and which has been shown to reverse the effects of SUMO attachment to MDM2, causing p53 stabilization (152). In addition to sumoylation, interaction of MDM2 with death-domain-associated protein (Daxx) and the deubiquitylating enzyme HAUSP can also alter MDM2 E3 liagase activity (153). HAUSP has been shown previously to stabilize MDM2 by promoting its deubiquitylation (154), whereas Daxx not only enhances HAUSP and MDM2 interaction by binding to both proteins, but also augments p53 ubiquitylation mediated by MDM2. However, under stress condition such as DNA damage, this complex formation is disrupted leading to Daxx dissociation followed by increase in MDM2 degradation and this probably contributes to stabilization of p53 (153). Enhanced degradation of p53 has been also observed upon MDM2 interaction with MTBP, while autoubiquitylation of MDM2 was diminished (57). In addition, it has been shown that interaction with MDMX, a structural homologue to MDM2, can also have a stabilizing effect on MDM2, whilst interaction with ARF has been shown to abrogate E3 ligase activity of MDM2 towards p53 (176,177).

MDM2 activity is also extensively regulated via phosphorylation, a posttranslational modification mediated by protein kinases. The "ataxia telangiectasia mutated" proteine kinase (ATM) has been reported to promote phosphorylation of

MDM2 at residue serine 395 resulting in reduced p53 degradation due to failure of nuclear export (89). Apart from phosphorylation by ATM, many different kinases have been implicated in multisite phosphorylation of MDM2, at least *in vitro*, such as: DNA activated protein kinase (DNA-PK), c-Abl protein tyrosine kinase, phosphatidylinositol 3-kinase (PI3-K/Akt), cyclin A-dependent kinases 1 and 2 (cyclinA-Cdk1/2) (reviewed in 75). Generally speaking, phosphorylation of MDM2 seems to abrogate its interactions with p53. For example reduced ability to form a complex with p53 has been observed upon MDM2 phosporylation by A-Cdk2 or DNA-PK in response to genotoxic stress (90,91). However, PI3-K/Akt pathway mediated phosphorylation of MDM2 on serine 168 and 186 residues seems to be essential for MDM2 translocation into the nucleus, which is necessary for MDM2's ability to interact with p53 and its function as a negative regulator of p53 (92).

Ubiquitylation is the most widely studied, however not the exclusive modification promoted by MDM2 E3 ligase activity ascribed to the RING finger domain of the protein. Another modification mediated by MDM2 is NEDDylation, which is conjugation of a small, ubiqutin-like protein called NEDD8 (135). Similarly to promoting self-ubiquitylation process, MDM2 is capable of promoting its own NEDDylation, however the precise role of that modification has not been elucidated. So far, only two other proteins have been reported to be modified by MDM2 this way: p53 and full-length (TA) p73. In both cases, transcriptional activity of targeted proteins is inhibited by MDM2-mediated NEDDylation (135,136).

1.3 MDM2 and human cancer

Mdm2 is an oncogene that is activated by increased expression, and not surprisingly, high levels of MDM2 have been observed in many different human tumours. MDM2 overexpression and/or amplification have been reported in, among others, soft tissue sarcomas (~30%) (32), gliomas (8 – 10%) (37), leukemias (~50%) (38), breast carcinomas (~70%) (39, 76), and malignant melanomas (~70%) (40, 78). It has also been demonstrated that MDM2 status can be a useful prognostic factor in a wide range of tumours. For example, high levels of MDM2 usually confer worse prognosis in, among others, breast carcinoma (76), leukemia (77) and soft tissue sarcoma (32) (reviewed in 73). However, in some cases there is a need for further studies, since observations from the same cancer type may be in contradiction indicating either worse or better prognosis as observed in case of soft tissue sarcoma, where studies on one cohort of samples revealed worse prognosis for patients expressing high levels of MDM2, while based on the other studies high levels of MDM2 conferred better prognosis for patients (32, 72).

In addition, it has been observed that high levels of both MDM2 and p53 occur in soft tissue sarcoma cells (32), bladder cancer (33), clear renal cell carcinoma (34) and these patients have significantly worse prognosis than patients with only high levels of either p53 or MDM2.

1.4 p53 – MDM2 interaction

p53 is one of the most important and widely studied tumor suppressor genes, since p53 mutation is the most frequent genetic alteration in human cancer

(24). p53 is a potent transcription factor for genes involved in inhibition of cell cycle (i.e. p21 WAF1/CIP1, cyclin G) and blood vessel formation (angiogenesis) (i.e. TSP1, BAI1), induction of apoptosis (i.e. BAX, GML), DNA replication and repair (i.e. GADD45), and cellular stress response (i.e. TP53TGI, CSR) (79). p53 is often called the "guardian of the genome", since its proper function seems to be indispensable for maintaining cellular genetic stability (23). In normal, unstressed cells p53 protein is maintained at a very low level. When a cell becomes stressed due to, for example, DNA damage or activation of oncogenes p53 is stabilized and thus it can perform its functions (24).

The first report to describe the existence of a p53 – MDM2 complex was published almost two decades ago where it was shown that MDM2 inhibits p53 function as a transcription factor (5). Studies mapping the interacting regions involved in formation of the MDM2 and p53 complex revealed that amino acids 25 – 109 on human MDM2 and 19 - 26 on p53 interact to form the complex. In addition, it has been shown that not only can MDM2 regulate p53 activity, but also p53 can regulate *MDM2* by promoting its transcription via the P2 promoter, as discussed earlier (26). Thus a negative autoregulatory feedback loop exists between p53 and MDM2, where active p53 stimulates expression of *MDM2* which in turn targets p53 for proteasome – dependent degradation and therefore this results in decreased p53 transcriptional activity, which leads to reduced levels of MDM2.

MDM2 regulation of p53 is crucial during development. It has been demonstrated that MDM2–null animals could not undergo normal embryogenesis and died during the first week of gestation; however the absence of both p53 and MDM2 allows normal development, suggesting that MDM2 is an essential negative

regulator of p53 during embryo development in mice (27, 80).

MDM2 is a key negative regulator of p53 that acts by inhibiting transcriptional activity of p53, but also by mediating ubiquitylation and proteasome - dependent degradation of p53 (81, 137). As described earlier in this chapter, MDM2 has an E3 ligase activity located on its RING finger domain at the C – terminal end and is capable of mediating both mono- and polyubiquitylation of p53. It has been shown that the presence of p300 might be necessary for MDM2-mediated polyubiquitylation of p53 leading to proteasome degradation (64,83,137). According to early studies, proteasome degradation of p53 was exclusively ascribed to cytoplasm, therefore MDM2-p53 nuclear export was considered to be indispensable for efficient p53 degradation (82, 85). However, there is a growing body of evidence suggesting that MDM2-mediated degradation of p53 can occur in the nucleus as well as in the cytoplasm, thus the nuclear export is not an absolutely required step in this process (86, 87).

Via its E3 ligase activity MDM2 also promotes NEDDylation of p53, resulting in inhibition of its transcriptional activity (135). Recent findings have revealed that ubiquitylation and NEDDylation mediated by MDM2, due to its E3 ligase activity, can be regulated independently of each other. One example comes from the studies on Tip60, transcription cofactor and MDM2 binding protein, which is able to inhibit MDM2 mediated NEDDylation of p53, while MDM2 ability to promote p53 ubiquitylation seems to be intact (138).

p53/MDM2 complex formation and function are regulated by several mechanisms such as phosphorylation of either p53, MDM2 or both; acetylation of p53; oligomerization of p53; and interactions with other proteins (75). In some cases

p53 and MDM2 are phosphorylated simultaneously by the same kinase (e.g. ATM) resulting in stabilization and restoration of p53 function due to release from inhibitory effect of MDM2-p53 complex (101). In addition, acetylation of p53 was shown to affect the interaction of MDM2 with p53-responsive promoters, allowing p53 to perform its transactivation function independently of its phospohorylation status (176). It has also been shown that oligomerization deficient p53 mutants have reduced affinity for binding to MDM2, therefore p53 oligomerization seems to be required for efficient binding to MDM2 and MDM2 mediated degradation (107).

1.5 p53 - independent function of MDM2

Oncogenic properties have been ascribed to MDM2 mainly on the basis of its interaction with p53, wherein MDM2 blocks p53 tumour suppression functions. However, a growing body of evidence supports the idea that MDM2 may contribute to cancer development through involvement in several cellular pathways in a p53-independent manner (18). Most of the well defined examples of p53-independent involvement of MDM2 in tumourigenesis are presented below:

- presence of high levels of both MDM2 (due to amplification/overexpression) and p53 (due to mutation) in several types of cancer including: soft tissue sarcomas and bladder cancers and confers worse prognosis (32,33); this shows that overexpressed and/or amplified MDM2 and mutated p53 may affect different pathways during carcinogenesis.
- overexpression of an MDM2 transgene in p53 null mice resulted in a higher rate of sarcomas in comparison to the incidence in p53 null mice (without

MDM2 transgene) (35); this study has provided *in vivo* evidence of MDM2-p53 – independent oncogenic mechanism/s.

- identification of around 40 different splice variants of MDM2 in tumours, that in most cases lack the p53 binding domain (20, 21); since they are unable to interact with p53, it is possible that they play p53-independent role/s in the cell. The example of the MDM2 – B variant, that has been shown to promote cell growth and inhibit apoptosis in a p53 independent manner, and also to induce tumour formation in transgenic mice supports this suggestion (36).
- overexpression of MDM2 in epithelial cells of murine mammary gland led to polyploidy in 30-45% of the cells and this was observed in both wild-type and p53 null mice. This observation suggests that overexpressed MDM2 might be involved in the initiation of DNA replication process (97).
- interaction between MDM2 and Nbs1 (Nijmegen breakage syndrome 1), a component of a DNA double strand break repair complex (Mre11-Rad50-Nbs1), suggests MDM2 p53-independent involvement in regulation of genome stability (175).
- interaction of MDM2 with a number of proteins involved in various cellular processes such as: cell cycle, differentiation, DNA synthesis, ribosome synthesis or transcription as shown in Figure 1.5.1 (18).

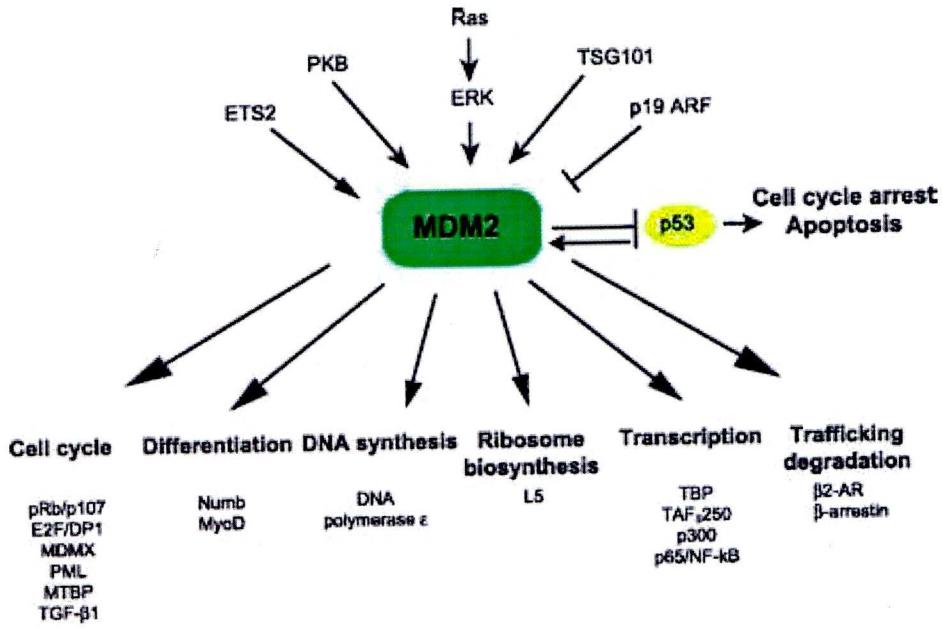


Figure 1.5.1 Diagram of MDM2 interacting proteins (figure reproduced from reference 2).

1.6 MDM2 interactions with other proteins

Apart from interaction with p53, MDM2 has been shown to interact with many others proteins involved in different pathways in a cell (Figure 1.5.1). These proteins usually either regulate (e.g. p19ARF, TSG101) or are regulated by MDM2 (e.g. E2F/DP1) or sometimes play both functions (e.g. p53).

In addition to the MDM2 interaction with p53 which has already been described, for the purpose of this thesis I will describe, in more detail, MDM2 interactions with key cell cycle regulators such as: E2F/DP1 and retinoblastoma protein (Rb), MDMX and interacting proteins discovered in our laboratory, such as: MDM2 binding protein (MTBP), dihydrofolate reductase (DHFR) and DNA polymerase ε.

- E2F1/DP1, which exists as a heterodimer, is a transcription factor for genes

involved in S-phase progression, whereas retinoblastoma protein (pRb), is a tumour suppressor that negatively regulates cell cycle by binding to E2F transcription factors. Upon phosphorylation of pRb, E2F1/DP1 is released and thus capable of performing its function as a transcription factor, leading to cell cycle progression. Interaction of MDM2 with pRb has been shown to inhibit its function as tumour suppressor, resulting in cell cycle promotion (53). Direct binding of MDM2 to E2F1/DP1 results in enhanced transcriptional activity and this effect is mediated independently of pRb (and p53) (98,99). Moreover, MDM2 was shown to prevent E2F1/DP1 degradation by binding to a domain engaged in E2F1/DP1 ubiquitylation, thus stabilizing this protein (155). Therefore, interaction of MDM2 with both E2F1/DP1 and/or pRB can lead to enhanced cell cycle progression (51,52,98,99).

- MDMX (aka MDM4) is structurally very similar to MDM2 based on observations that MDMX contains a RING finger-like domain (but unlike MDM2 does not possess E3 ligase activity), nucleolar localization signal (NoLS) within RING domain and p53-binding domain at the amino-terminal end. Similarly to MDM2, MDMX has been shown to interact with p53 and inhibit its transcriptional activity (54), mostly by direct interaction. Studies performed using yeast two – hybrid system and *in vitro/in vivo* binding assays have demonstrated that MDM2 and MDMX interact with each other via their RING finger domains located at C – terminal end of each protein. Co–expression of MDMX and MDM2 in 293 cells resulted in suppression of proteasome dependent degradation of MDM2, suggesting that MDMX has a stabilizing effect on MDM2 (55).

Research performed in our laboratory using yeast – two hybrid screen has revealed novel MDM2 interacting proteins: MTBP and DNA polymerase ε (56 – 59),

whilst a proteomics-based approach led to the discovery of DHFR as a MDM2 binding protein (134).

DHFR is a vital component of the cell machinery involved in a number of important processes, including DNA synthesis. MDM2 seems to interact with DHFR and this interaction results in monoubiquitylation of DHFR, which requires an intact RING finger domain of MDM2. As a result of monoubiquitylation, the activity of DHFR is inhibited by MDM2 in a dose dependent manner, while DHFR stability remains undiminished. It should be noted that most of the experiments in these studies were performed in a p53 null background and therefore this interaction may be added to the list of p53-independent functions of MDM2 (134).

MTBP has been shown to bind to MDM2 region between 167 and 304 aa by its carboxy–terminal 380 amino acids domain. It has been demonstrated that MTBP has the ability to induce a p53 – independent G1 arrest and that, this effect can be overcome by MDM2 (56). Further studies on MTBP function have suggested that it regulates the ubiquitin ligase activity of MDM2. It seems to enhance MDM2 – mediated ubiquitylation and degradation of p53, but inhibits autoubiquitylation of MDM2 leading to MDM2 stabilization (57).

DNA polymerase ε is a DNA polymerase implicated in several essential processes in the cell specifically replication, DNA repair and checkpoint control (29). It has been shown that the carboxyl – terminal region of DNA polymerase ε catalytic subunit is required for the interaction with the amino – terminal region of MDM2 (between aa 50 – 166) (58). Moreover, human MDM2 expressed in insect cells as well as in *Escherichia coli* has the ability to stimulate DNA polymerase ε activity and regions involved in protein interaction are indispensable for this stimulation (59).

1.7 Eukaryotic DNA polymerases – general information

DNA polymerases are enzymes that play a key role in processes that are vital for cell survival and proliferation, such as DNA replication and repair. Polymerases catalyse phosphoryl transfer reactions, and in order for the polymerization reaction to occur cooperation with two metal ions (usually magnesium-Mg^{2+}), that stabilize the formation of phosphodiester bond between 3'hydroxyl (3'OH) group of the primer and incoming nucleotide as well as conformational change of the enzyme itself are all necessary (29,164). Most DNA polymerases have a specific conserved structure, which resembles a right hand with palm, fingers and thumb, as presented in Figure 1.7.1. The catalytic site of the enzyme is located on the "palm" and by moving the "fingers" subdomains form a fist shape which creates a binding space for a new nucleotide (29,165).

DNA polymerases can be distinguished partly by features such as processivity and fidelity which vary significantly among different polymerases (28,173). The latter defines the accuracy with which a polymerase adds nucleotides based on template sequence, whilst processivity can be defined by the number of nucleotides added by a DNA polymerase per interaction with the 3'OH-end of the primer (28). It seems that processivity might be correlated with the function that a particular polymerase performs in the cell. For example, DNA repair polymerases are in general characterized by lower processivity than polymerases involved in replication (28,174). In terms of fidelity, some polymerases possess intrinsic exonuclease activity (3'→ 5'), which allow them to correct their own mistakes by excision of an incorrect nuclotide and replacing it with a correct one (28).

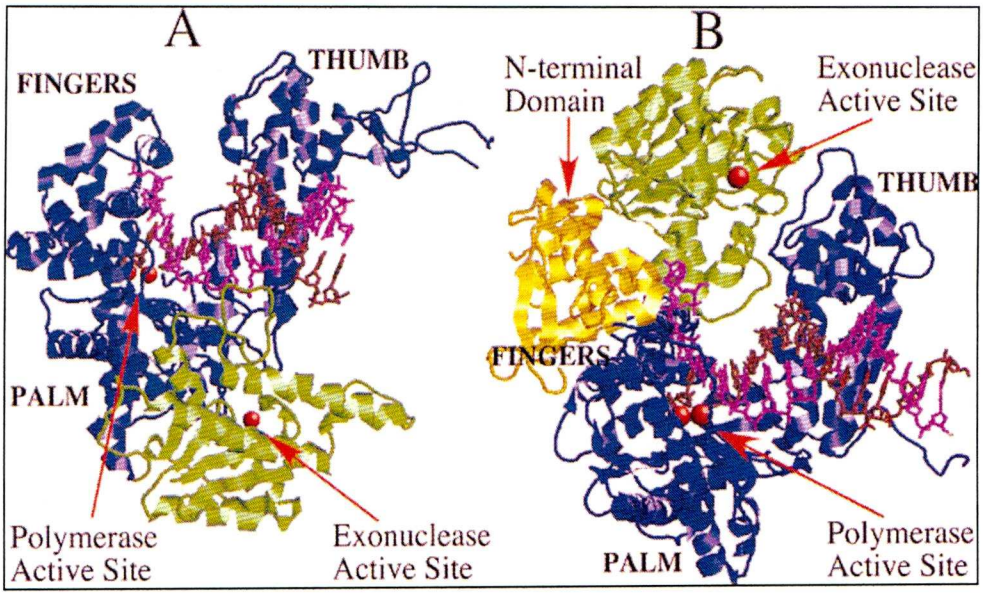


Figure 1.7.1 Structure of DNA polymerase. "Right hand" structure of bacteriofage T7 DNA polymerase representing polymerases class A (A) and bacteriofage RB69 DNA polymerase representing class B DNA polymerases (B) with a dideoxy-terminated primer-template DNA and an incoming base-paired dNTP (figure reproduced from reference 42).

There are around 20 polymerases that have been described in eukaryotic cells (including terminal transferase and telomerase) (reviewed in 29). Based on the presence of conserved regions eukaryotic DNA polymerases can be divided into four classes/families (6), Table 1.7.1 shows the organization of polymerases into classes and their respective functions.

The general function of each class of polymerases can be described as follows: class B consists of polymerases responsible mainly for replication, class X consists of polymerases required for DNA repair, class Y gathers together translesion synthesis polymerases (TLS) and class A includes replicative (pol γ mitochondrial replication) and repair polymerases (θ) (Table 1.7.1) (29). However, in most cases

one polymerase may be involved in more than one functional assignment within a cell. One such example are B class polymerases, which are primarily involved in DNA replication, wherein DNA polymerase α initiates this process by synthesizing primers on the leading as well as lagging strand and further DNA elongation as a process that is taken over by DNA polymerases ε and δ, respectively (42). Apart from involvement in DNA synthesis, eukaryotic polymerases belonging to class B can also take part in DNA repair, checkpoint control and telomere maintenance (29).

CLASS	POLYMERASE	FUNCTION
B	α	DNA replication
	δ	
	ε	
	ζ	
X	β	DNA repair
	λ	
	μ	
	σ	
Y	η	Translesion synthesis
	ι	
	κ	
	Rev I	
A	γ	DNA replication and repair
	θ	
	Other	

Table 1.7.1 Classes of eukaryotic DNA polymerases.

1.8 Structure and biological function of DNA polymerse α

DNA polymerase α belongs to class B of the DNA polymerase family (6). It consists of a heterotetrameric complex that exhibits DNA polymerase activity as well as RNA polymerase activity and has a unique ability to initiate DNA

synthesis *de novo*. Compared to other polymerases involved in the replication process, such as polymerase δ and ε, DNA polymerase α processivity is lower. In addition, DNA polymerase α does not possess intrinsic 3'→5' exonuclease activity, thus the enzyme is not capable of correcting its own mistakes introduced during DNA synthesis (28). The error rate of DNA polymerase α, however, can be diminished via the extrinsic exonuclease activity of DNA polymerase δ (166), and the fidelity of DNA polymerase α also seems to be enhanced via interaction with replication protein A (RPA) which seems to function partly to prevent DNA polymerase α from introducing incorrect nucleotide (69).

		Organism			
Subunit		*Sacharomyces cerevisiae* (102)	*Drosophila melanogaster* (104)	*Mus musculus* (9)	*Homo sapiens* (103)
Catalytic subunit		167	182	180	180
B subunit		79	73	68	68
Primase	large	62	61	54	58
	small	48	50	46	48

Table 1.8.1 Molecular weights (kDa) of DNA polymerase α in different eukaryotic organisms.

DNA polymerase α is composed of four subunits and the molecular weights of all four subunits vary slightly between different eukaryotic organisms (examples are presented in Table 1.8.1) (29). Despite the small differences in molecular weights, the functions ascribed to each particular subunit of DNA

polymerase α seem to be the same in all eukaryotic organisms (29). Polymerase enzymatic activity is localized within the largest subunit called the catalytic subunit whilst the primase consists of the two smallest subunits of the complex. The fourth subunit, called the B subunit, does not seem to have any enzymatic activity and most likely has a regulatory and structural role (7, 8, 71).

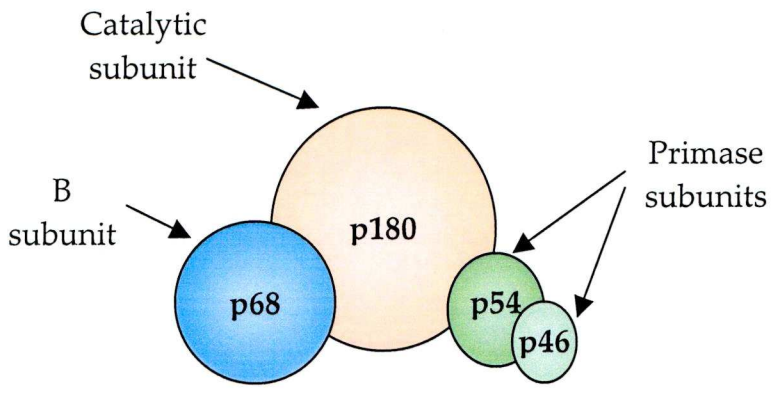


Figure 1.8.1 Diagram of murine DNA polymerase α complex (based on information provided by reference 9).

As stated already DNA polymerase α is a multisubunit enzyme, therefore in order to better understand its functioning and potential interactions with other proteins and factors within a cell, it is important to establish the order of assembly and structure of the complex. Studies on interactions between subunits during their assembly have been performed by co-expressing all components of the murine DNA polymerase α complex in COS – 1 cells (9). All reported components of DNA polymerase α complexes comprising either all four subunits, three subunits: p180/p68/p54, or heterodimers of p180/p68 and p54/p46 can be translocated into the nucleus. It should be noted however that for complexes consisting of three subunits:

p68/p54/p46 or p180/p68/p46, specifically p68 and p46, respectively, remained in the cytoplasm, whilst dimers p54/p46 and p180/p68 were translocated to the nucleus. It has also been reported that nuclear localization sequences (NLS) are present on both p180 and p54 subunit. According to these findings, it has been suggested that p68 and p54 bind directly to the p180 subunit and that p46 binds to the complex via p54 (Figure 1.8.1). Several regions present in the p54 (p58 in human, see Table 1.8.1) sequence have been shown to be involved in interactions with the smaller primase subunit (30). Similar results were obtained for the B subunit, which interacts with p180, but interacting regions are distributed throughout the protein (41).

p180, the catalytic subunit of DNA polymerase α has been analysed in more detail. Studies carried out on murine DNA polymerase α have revealed that the catalytic subunit contains three domains as shown in Figure 1.8.2. The minimal region required for DNA polymerase activity lies between aa 330 and 1279 and is called the core domain, whilst both amino (1-329 aa) and carboxy-terminal (1280-1465 aa) domains are dispensable for polymerase activity (9). With regard to subunit assembly, only the carboxy–terminal domain of the catalytic subunit has been described to be involved in interaction with p68 and p54 subunits. There are several conserved regions within the catalytic subunit: six regions (from I to VI) seem to be highly conserved among class B polymerases (prokaryotic and eukaryotic), and another five regions (A-E) are conserved in eukaryotic DNA polymerases α (167,168). Highly conserved regions are located in different parts of a hand shape structure (Figure1.7.1) and are involved in performing crucial activities/functions of the enzyme. For example, regions I and II located on the palm subdomain, along with region III on the fingers subdomain seem to be involved in deoxynucleotide

triphosphate (dNTP) binding, whereas region V, located on the thumb, seems to play a role in DNA binding and enzyme processivity (29, 164, 169).

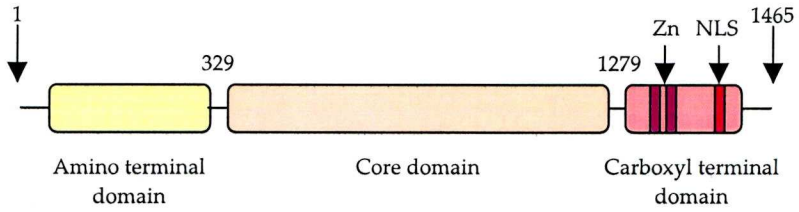


Figure 1.8.2 Structure of murine DNA polymerase α catalytic subunit (p180). Core domain harbours polymerase activity, while carboxyl terminal domain is engaged in subunits assembly; Zn – zinc finger region, NLS – nuclear localization signal (based on information provided by reference 9).

The main function of DNA polymerase α is DNA polymerization during DNA replication. The enzymatic activity of DNA polymerase α is carried by the largest, catalytic subunit, whereas the heterodimeric complex of the smallest subunits of DNA polymerase α possesses primase activity and has a unique ability to initiate DNA synthesis *de novo* (8). DNA polymerase α, due to its primase activity (DNA directed RNA polymerase) is capable of synthesizing short (7 – 10 ribonucleotides) RNA primers on both leading and lagging strands (Okazaki fragments) during DNA replication (10). Primase catalytic activity is localized entirely within the smallest subunit, however the presence of the second subunit of the primase complex is indispensable for its proper function and required for both primer initiation and elongation (11).

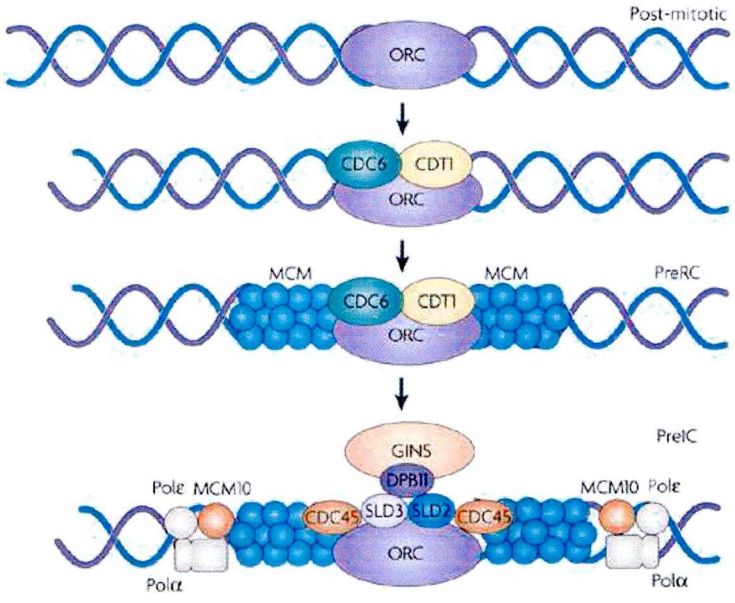


Figure 1.8.3 Assembly of the pre-replicative and pre-initiation complexes (figure reproduced from reference 196).

In order to perform its catalytic functions during DNA synthesis DNA polymerase α needs to be recruited to an origin of replication. However, several events need to take place prior to enzyme loading (as shown in Figure 1.8.3), starting with recognition of replication origin by origin recognition complex (ORC). ORC serves as a binding platform for cell division cycle 6 (Cdc6) and chromatin licensing/DNA replication factor 1 (Cdt1) that are required for subsequent loading of a minichromosome maintenance 2-7 proteins (MCM2-7), which completes the formation of the pre-replicative complex (pre-RC) during the G1 phase of the cell cycle (42). During the next step, S phase-promoting cyclin-dependent kinases (CDKs) and Dbf4-dependent kinase (DDK) mediate recruitment of minichromosome maintenance protein 10 (Mcm10), cell division cycle protein 45 (Cdc45), GINS, Dpb11, Sld 2 and 3 proteins, forming the pre-initiation complex (pre-IC) (114). Upon

transition to pre-IC the helicase activity of the CMG complex, consisting of Cdc45, GINS and Mcm2-7, is triggered leading to origin DNA unwinding (125,126). Replication protein A (RPA) is loaded onto single stranded DNA and along with Cdc45 and Mcm10 recruits DNA polymerase α, and this initiates DNA replication by synthesizing oligomeric-ribonucleotide primers (119, 120). RNA primer synthesis involves five major steps: initially DNA polymerase α binds to a template (single stranded DNA) followed by binding of two nucleotide triphosphates (NTPs), first of which is usually purine (12). During the next step NTPs are polymerized to create a RNA primer of 7- 10 oligoribonucleotides in length. The mechanism involved in primer nucleotide "counting" is still unclear, however it has been shown that the larger subunit of the primase complex may play a role in this process (11). After a RNA primer of defined length is synthesized (typically c. 10 nuclotides), it is transferred to the DNA polymerase active site where approximately 20 DNA nucleotides are added (12). When the RNA/DNA primer of approximately 30 nucleotides is synthesized on the leading strand, as well as on the lagging strand (Okazaki fragments), DNA polymerase α seems to be replaced by polymerases ε and δ respectively, in order to continue the process of DNA synthesis (42).

Even though the main function of the DNA polymerase α complex is the initiation of DNA synthesis during DNA replication, it also seems to be involved in cell cycle regulation (66,67), telomere maintenance (68) and epigenetic control (156,160).

A few early reports suggested that primase subunits of DNA polymerase α, in addition to being capable of RNA primer synthesis, may also be indispensable for activation of the S/M checkpoint of the cell cycle (66). It has been demonstrated

that abrogation of primase activity, either due to inhibition or mutation results in failure to induce Chk1 phosphorylation (66,163). This is important because Chk1, a checkpoint kinase, becomes phoshorylated by ATR (ataxia telangiectasia mutated and Rad3-related protein kinase) upon DNA damage (162).

There is a growing body of evidence suggesting that DNA polymerase α is also involved in telomere maintenance. Telomerase is the main enzyme responsible for synthesis of the leading strand (G-rich) at the end of chromosomes, however complementary strand synthesis requires a different mechanism to be engaged (159). DNA polymerase α seems to contribute to this process by interacting with proteins involved in telomere maintenance and also by synthesizing the C-rich strand (lagging strand) of telomeric DNA (68, 156-8).

Results obtained from experiments performed with *Sacharomyces cerevisiae* arrested in M phase, have suggested that CDC17 and Pri2 (yeast DNA polymerase α and primase, respectively) are required not only for telomere lagging strand synthesis, but also to coordinate telomerase mediated leading strand duplication (68). These findings have been further supported by experiments with CDC17 temperature-sensitive mutants showing that proper regulation of telomere lengthening was disrupted upon CDC17 mutation (156). Moreover, an interaction between the catalytic subunit of DNA polymerase α and telomere binding protein Cdc13p has been observed in yeast, which could explain how the DNA polymerase might be recruited to telomeres (157). Apart from the catalytic subunit, the B subunit (Pol12 in yeast) of DNA polymerase α has been described to interact with a protein involved in telomere protection and length regulation known as Stn1 (158). Mutations in either catalytic or B subunit of DNA polymerase α can cause abnormal

telomere elongation, suggesting that this subunit of the enzyme also has a role in telomere maintenance (157,158).

Along with the role in telomere maintenance in yeast, a potential role for DNA polymerase α in epigenetic control has been discovered (156). It has been shown that transcriptional silencing in telomeres as well as in centrosomes and the mating-type region of fission and budding yeast was lost in CDC17 (yeast catalytic subunit of DNA polymerase α) yeast mutants (156,160). Epigenetic control of gene regulation by DNA polymerase α might be mediated by the enzyme interaction with Swi-6 chromodomain protein, since localization of Swi-6 protein at silenced loci was abrogated in DNA polymerase α mutants displaying silencing defects (160,161).

DNA polymerases play central roles in processes that control genome integrity (DNA replication and repair and cell cycle checkpoint control) and it is therefore not surprising that they are also implicated in cancer (170). In particular, overexpression of specialized polymerases such as β, λ and ι has been found in number of cancer samples (105, 170). In addition, a greater than two – fold increase in DNA polymerase α expression levels has been observed in tumour cells compared to normal tissue (105). Since DNA polymerase α is an indicator of cell proliferation, it is unclear whether its overexpression is just a consequence or rather an independent cause of carcinogenesis.

Additional studies, supporting a role for DNA polymerase α in cancer development, have demonstrated that dehydroaltenusin, a specific inhibitor of DNA polymerase α activity (originally obtained from fungus (*Alternaria tennuis*)) seems to have anti-tumour properties. Growth of human cancer cells in culture, as well as proliferation of tumour cells *in vivo* have been shown to be inhibited upon addition

of dehydroaltenusin, which appeared to interact with the catalytic subunit of DNA polymerase α (106,172). Based on these studies, it is conceivable that dehydroaltenusin may be the first of a new family of potential anti-cancer agents that target DNA polymerases.

1.9 Regulation of DNA polymerase α activity

DNA replication is one of the most important processes in a cell and therefore needs to be tightly regulated at each step. The activity of DNA polymerase α, the only enzyme capable of initiating DNA synthesis *de novo*, is controlled by interactions with a number of cellular proteins and factors. Some of the interactions between DNA polymerase α and proteins taking part in formation of pre-replication/initiation complex and progression of the replication fork have already been briefly described above and some of these are described in more detail below (42).

Minichromosome maintenance protein 10 (Mcm10)

After formation of the pre-replicative complex consisting of ORC, Cdc6, Cdt1 and Mcm2-7, Mcm10 is recruited to the origin of replication (42). Mcm10 is involved in loading of DNA polymerase α onto DNA along with cell division cycle protein 45 (Cdc45) and replication protein A (RPA) (119, 120). Apart from the physical interaction with DNA polymerase α (43, 120), it has been demonstrated that Mcm10 can also stimulate DNA polymerase α activity *in vitro* (43). Moreover, it has been shown that in the absence of Mcm10, the catalytic subunit of DNA polymerase

α is destabilized and DNA synthesis is inhibited, whereas the absence of DNA polymerase α only delays the replication (121,122). It seems that Mcm10 somehow "recycles" DNA polymerase α activity and in addition it also contains primase activity on its own (123).

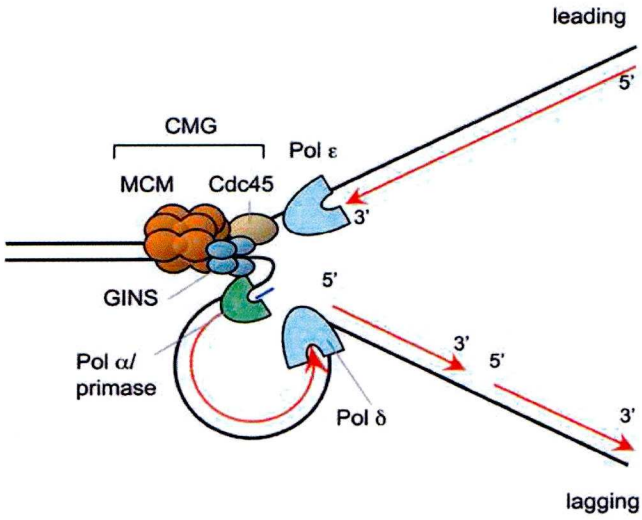


Figure 1.9.1 Schematic representation of the replication fork
(figure reproduced from reference 125).

GINS (Go, Ichi, Nii, San-Japanese; five, one, two, three-English)

Even though it has been discovered only recently, the GINS protein complex appears to be a vital factor in the DNA synthesis process. This protein complex is highly conserved in eukaryotes, and is also present in archaea (118,124). The main roles ascribed to GINS are initiation and progression of DNA replication (124,125). It has been shown that in the presence of GINS, minichromosome maintenance proteins (MCMs) can interact with cell-division cycle protein 45 (Cdc45) and perform helicase function (125, 126). These proteins actually form a CMG (Cdc45-

Mcm2-7-GINS) complex, that seems to be indispensable for progression of the replication fork (125, 127, 128) (Figure 1.9.1).

The role of GINS in regulation of DNA polymerase α activity has been studied *in vitro,* where it has been demonstrated that recombinant human GINS (hGINS) complex can interact with primase subunits and stimulate polymerase activity of DNA polymerase α (129). Despite physical interaction between GINS and DNA polymerase α, GINS complex does not seem to be involved in loading of the enzyme onto the chromatin. Curiously, inactivation of one of the four components of the GINS complex has been shown to abolish DNA polymerase ε loading, whereas it does not affect the loading of DNA polymerase α (130), which is in accordance with previous findings (119, 120).

Cell division cycle protein 45 (Cdc 45)

Studies carried out on budding yeast have demonstrated that Cdc45, by interacting with minichromosome maintenance proteins (MCMs), takes part in initiation of DNA replication (131). In addition, it has been shown that Cdc45 isolated from *Xenopus* egg extracts is responsible for loading the DNA polymerase α complex onto chromatin, but this action also required the activity of cyclin-dependent kinases (CDKs) engaged in S - phase (50). Furthermore, results from experiments using human proteins have demonstarted that Cdc45 can interact with the p68 subunit of DNA polymerase α and Mcm7 protein, supporting above mentioned findings (49).

In the light of more recent results, loading of DNA polymerase α onto

chromatin is not only dependent on Cdc45, protein but also on the action of Mcm10 and RPA (119, 120).

Replication protein A

Replication protein A (RPA) is a heterotrimeric protein, consisting of subunits of approximately 70kDa, 30kDa and 14kDa (44). RPA binds to single – stranded DNA via its largest subunit. Using the simian virus 40 (SV40) model of replication it has been demonstrated that RPA is required for the formation of the primosome in which a complex containing T antigen and RPA is recognized by DNA polymerase α. Binding occurs between primase subunits of DNA polymerase α and the 70kDa subunit of RPA and this interaction seems to be essential for initiation of the replication process of SV40 (45).

In addition, the 70kDa subunit of RPA seems to stimulate DNA polymerase α activity *in vitro* (46,132). This effect is mediated by a region of 327 aa located at the amino-terminal end of the 70kDa subunit whilst a region between aa 169 and 327 has been shown to increase the processivity of DNA polymerase α (132). Moreover, RPA seems to function as a "fidelity clamp" stabilizing the DNA polymerase α complex, and since this enzyme does not have 3'→5' exonuclease activity this reduces the number of mistakes introduced by DNA polymerase α (69).

T antigen

T antigen is a helicase involved in DNA unwinding and together with host proteins plays an important part in SV40 replication. Interaction between DNA polymerase α and T antigen has been identified during studies on proteins participating in a replication process with T antigen (47). It has been shown that the p180 subunit of DNA polymerase α is sufficient for complex formation with T antigen (45) and that T antigen enhances enzymatic activity of DNA polymerase α (48).

p53

Using Biomolecular Interaction Analysis (BIA) based on surface plasmon resonance, it has been shown that p53 can form a complex with heterotetrameric DNA polymerase α, as well as with a heterodimer composed of the two largest subunits of DNA polymerase α. In order to determine the region of p53 that is responsible for binding to DNA polymerase α, a series of p53 deletion mutants was examined and this showed that the carboxy-terminal domain of p53 plays a role in forming stable interactions with the enzyme. In addition, it has also been shown that p53 and T antigen compete for binding to DNA polymerase α (13).

Further studies performed on CEM cells confirmed the presence of p53 – DNA polymerase α complex in human cells (14). Moreover, since p53 has been described to have a 3'→5' exonuclease activity, question arose whether this function has an application with regards to the DNA polymerase α complex function. It has

been shown that a p53-DNA polymerase α complex was able to cut unpaired 3' nucleotides and elongate the strand. However, due to the low velocity of this reaction, p53 activity has not been considered as an external proofreading exonuclease for DNA polymerase α (14).

Retinoblastoma protein (Rb)

Retinoblastoma protein (Rb) is a well known tumour suppressor that inhibits cell cycle progression mainly via interactions with members of the E2F family of transcription factors. The hypophosphorylated form of Rb is an active form of the protein capable of performing its inhibitory functions, whereas the phosphorylated form can no longer bind to E2F, thus allowing the cell cycle progression. It has been described, based on immunoprecipitation experiments, that DNA polymerase α can form a complex with Rb in Raji cells (15). Rb interacts with the catalytic subunit of heterotetrameric DNA polymerase α complex. Furthermore, phosphorylated form of Rb has been shown to increase the rate of switching from RNA to DNA, stimulate DNA polymerase α activity and enhance its processivity (16).

In addition to protein-protein interactions, DNA polymerase α activity can also be regulated by phosphorylation as described below.

Phosphorylation of DNA polymerase α - primase

The activity of a number of components of replication machinery, including DNA polymerase α are regulated by phosphorylation (109,110,111,112,114). The DNA polymerase α heterotetrameric enzyme is phosphorylated only on its two

biggest subunits – the catalytic subunit (p180 in mammals) (Thr174, Ser209, Thr219) and structural B subunit (Ser141, Ser147, Ser152, Thr156) (109,116). Cyclins A and E seem to play a key role in the phosphorylation of DNA polymerase α, and as presented in Figure 1.9.2 their activity peaks reflect their involvement in progression to particular phase during cell cycle (110,111,112,133).

Phosphorylation of DNA polymerase α has been described to occur in a cell cycle-dependent manner (70, 71, 113). When the enzyme is actively engaged in the DNA replication process it seems to be less phosphorylated i.e. during G_1/S transition, whereas it is more phosphorylated in late S phase and also G2/M transition (71, 116). It is noteworthy that the DNA polymerase α form active during G1/S phase also seems to have higher affinity for single stranded DNA than the "more" phosphorylated mitotic enzyme (71).

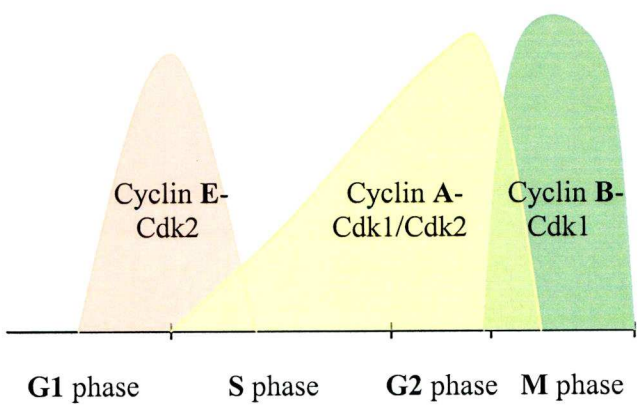


Figure 1.9.2 Schematic representation of activity of selected cyclins during cell cycle. Cyclin E-Cdk2 is involved in a progression from G1 to S phase, cyclin A-Cdk1/2 is active mainly in S – phase to ensure its completion and cyclin B – Cdk1 triggers M phase (figure reproduced from reference 133).

Moreover, DNA polymerase α activity seems to depend on which particular cyclin has been involved in the modification (70, 116). As an illustration, initiation

of SV40 replication seems to be inhibited upon phosphorylation of DNA polymerase α with cyclin A-Cdk2, but not with cyclin E-Cdk2 kinase (70, 115, 116, 117). More importantly, it appeared that depending on the cyclin involved in phosphorylation (either E or A), DNA polymerase α-primase can form two distinct subpopulations which can be distinguished using two different antibodies (116). The hypophosphorylated subpopulation associated with cyclin E-Cdk2 (and protein phosphatase 2A-PP2A) appears in G_1, whilst more phosphorylated subpopulation associated with cyclin A–Cdk2 is present during S and G_2 phase of the cell cycle (116). Based on the visualization of the active DNA synthesis sites by BrdU staining, colocalization and immunoprecipitation studies with MCM2 protein the hypophosphorylated form of DNA polymerase α-primase is thought to be involved in initiation of DNA replication on the leading strand, whereas phosphorylated enzyme is engaged in primer synthesis on the lagging strand (116). It appeared that different phospohorylation status of DNA polymerase α has an effect on the activity and function of the enzyme in the cell.

AIMS OF THIS STUDY

After many years of research on MDM2, it is clear that the primary oncogenic potential of this protein is mainly displayed via its ability to negatively regulate p53. MDM2 binds to p53 and in doing so inhibits its transactivation function, and in addition down-regulates p53 by promoting ubiquitylation and subsequent proteasome dependent degradation. However, a growing body of evidence indicates that MDM2 in addition to functions related to p53 manifests p53-independet activities. Since MDM2 protein – protein interactions seem to play the most important role in determining its activities, our research group has focused on identifying MDM2 binding partners and then studying the significance of such interactions for cell function. During the course of studies on the relationship between MDM2 and DNA polymerase ε performed by members of our research group, it was discovered that MDM2 co-purifies with DNA polymerase α, and this served as the basis for the studies performed in this thesis. In order to further investigate the interaction between MDM2 and DNA polymerase α, a key enzyme engaged in DNA synthesis , we have performed experiments to:

- determine whether MDM2 interacts directly with DNA polymerase α catalytic subunit (p180),
- map regions involved in the interaction between MDM2 and catalytic subunit of DNA polymerase α (p180) by performing *in vitro* binding assay and co - immunoprecipitation experiments with MDM2 deletion mutants,

- study the effect of MDM2 on DNA polymerase α enzymatic activity *in vitro* utilizing DNA polymerase assay (MDM2 stimulates the enzymatic activity of DNA polymerase ε and both polymerases are members of the B family of polymerases),
- examine the ability of MDM2 E3 ligase activity to promote p180 modifications,
- examine MDM2 and DNA polymerase α interactions *in vivo* taking into account existence of two different subpopulations of DNA polymerase α depending on its phosphorylation status.

Performing these experiments should allow us to gain insight into the significance of the MDM2–DNA polymerase α catalytic subunit (p180) interaction in cells and begin to ask key questions regarding the potential contribution of this to S–phase promotion, a candidate mechanism through which MDM2 might mediate some of its p53–independent oncogenic effects.

CHAPTER II

MATERIALS AND METHODS

2.1 Plasmids and constructs

pQE32 Mdm2 encodes full length murine 6xHis–tagged MDM2; used for expression and purification of protein from bacterial cells, pQE32 vector is commercially available from Qiagen.

pQE30 amino (N-) terminal half of Mdm2 encodes approximately 246 amino terminal amino acids of human 6xHis–tagged MDM2; used for expression and purification of protein from bacterial cells, pQE30 vector is commercially available from Qiagen.

pQE32 carboxy- (C-) terminal half of Mdm2 encodes approximately 252 carboxy-terminal amino acids of human 6xHis–tagged MDM2; used for expression and purification of protein from bacterial cells, pQE32 vector is commercially available from Qiagen.

pQE31 Δ166Mdm2 encodes murine 6xHis-tagged MDM2 lacking 166 amino terminal amino acids; used for expression and purification of protein from bacterial cells, pQE31 vector is commercially available from Qiagen.

pQE31 p53 encodes full length human p53; used for expression and purification of protein from bacterial cells, pQE31 vector is commercially available from Qiagen.

pBTBc601c encodes a 6xHis–tagged C–terminal variant of the apical domain of the mycobacterial heat shock protein CPN60.1; used for expression and purification of protein from bacterial cells, construct was kindly provided by Dr. Peter Tormay.

pBlueBacHis2p180 encodes catalytic subunit of 6xHis–tagged human DNA polymerase α; used for expression and purification of protein from insect cells, pBlueBacHis2 baculovirus transfer vector is commercially available from Invitrogen.

pSK-BBV FL p180 encodes full length of human p180 - catalytic subunit of DNA polymerase α; used for *in vitro* transcription/translation under control of T7 promoter, derivative of pBluescript SKII+ (commercially available from Stratagene) modified by Dr. Mark Boyd and Dr. Nikolina Vlatković.

pSK-BBV N-terminal half of p180 encodes approximately 800 amino terminal amino acids of human p180 - catalytic subunit of DNA polymerase α; used for *in vitro* transcription/translation under control of T7 promoter, derivative of pBluescript SKII+ (commercially available from Stratagene) modified by Dr. Mark Boyd and Dr. Nikolina Vlatković.

pSK-BBV C-terminal half of p180 encodes approximately 700 carboxy-terminal amino acids of human p180 - catalytic subunit of DNA polymerase α; used for *in vitro* transcription/translation under control of T7 promoter, derivative of pBluescript SKII+ (commercially available from Stratagene) modified by Dr. Mark Boyd and Dr. Nikolina Vlatković.

pSK-BBV 65 encodes carboxy-terminal half of DNA polymerase ε catalytic subunit; used for *in vitro* transcription/translation under control of T7 promoter, derivative of pBluescript SKII+ (commercially available from Stratagene) modified by Dr. Mark Boyd and Dr. Nikolina Vlatković.

pBBV MDM2 encodes full length human MDM2; used for *in vitro* transcription/translation under control of T7 promoter; construct was kindly provided by Dr. Dale Haines.

pGEX2TK-GSTMdm2 encodes full length human Glutathione S-transferase (GST) tagged MDM2; used for expression and purification of protein from bacterial cells, pGEX2TK vector is commercially available from GE Healthcare.

pGEX-6P-1 GST encodes Glutathione S-transferase; used for expression and purification of protein from bacterial cells, pGEX-6P-1 vector is commercially available from GE Healthcare.

pCMVNeoBam Mdm2 encodes full length human MDM2; used for expression in mammalian cells, construct was kindly provided by Dr. Bert Vogelstein.

pCMVNeoBam Δ49 Mdm2 encodes human MDM2 lacking of 49 amino terminal amino acids; used for expression in mammalian cells.

pCMVNeoBam3 Cys464Ala Mdm2 encodes RING finger mutant of human MDM2; used for expression in mammalian cells.

pCMVHA 166, 205, 230, 280, 335 Mdm2 encode respectively, amino terminal 166, 205, 230, 280, 335 amino acids of human hemagglutinin (HA) tagged MDM2; used for expression in mammalian cells, pCMVHA vector is commercially available from Clontech.

pcDNA3 NEDD8 encodes full length human NEDD8; used for expression in mammalian cells, construct was kindly provided by Dr. Dimitris Xirodimas.

pBluescript SK $^-$ His Ubiquitin encodes six human ubiquitins; used for expression in mammalian cells.

His Ubiquitn mutant Lys29,48,63Arg construct was kindly provided by Dr. Sylvie Urbe.

pBluescript SK $^-$ HA Ubiquitin encodes eight human ubiquitins; used for expression in mammalian cells.

pET32ap180 encodes full length human p180 – catalytic subunit; pET32a vector is commercially available from Novagen.

Cloning of pCMVHA Δ166 Mdm2 which encodes human MDM2 lacking amino terminal 166 amino acids. Following primers were used to amplify the fragment from pCMVNeoBam Mdm2 template and introduce KpnI restriction endonuclease site:

5' Mdm2 KpnI primer (MWG) – 5'gagaggtaccgagacagaagaaaattcagatgaa3';

3' Mdm2 KpnI primer (MWG) – 5'gagaggtaccctaggggaaataagttagcacaatc3'.

The amplified fragment was ligated into a pCR2.1-TOPO cloning vector (Invitrogen) and sequenced (MWG) to check for mutations that may have been introduced by the PCR. The correct Δ166 Mdm2 fragment was then subcloned into pCMVHA vector (Clontech) using KpnI restriction endonuclease (New England Biolabs Inc.), construct has been used for expression in mammalian cells.

Subcloning of p180 – catalytic subunit of DNA polymerase α into **pCEP4 vector** (Invitrogen). The p180 fragment (without TAGs) was excised from pET32a p180 construct with KpnI and subcloned into KpnI site of pCEP4 vector, construct has been used for expression in mammalian cells.

2.2 Antibodies

Primary antibodies

- **N-19** (Santa Cruz Biotechnology Inc.) is a goat polyclonal antibody against **DNA polymerase α** that has been raised using a fragment of human DNA polymerase α localized close to N – terminus of the enzyme. This antibody was used both: in western blotting and immunoprecipitation experiments.
- **HP180-12** and **SJK 132-20** (a kind gift from Professor Irene Dornreiter) are mouse monoclonal antibodies against **p180** (catalytic subunit of DNA polymerase α). SJK 132-20 preferentially detects the phosphorylated form of DNA polymerase α, whilst HP 180-12 exclusively binds to either non- or hypophosphorylated form of DNA polymerase α.
- **PRIM1** (Aviva Systems Biology) is rabbit polyclonal antibody against **primase 49kDa subunit** that recognizes region of human PRIM1 with an internal ID of P26856.
- **PRIM2** (Aviva Systems Biology) is rabbit polyclonal antibody against **primase 58kDa subunit** that recognizes region of human PRIM2 with an internal ID of S24607.
- **Ab-1** (clone IF2, Oncogene) is a mouse monoclonal antibody against **MDM2** that recognizes an epitope between amino acids 26 and 169 of human MDM2 protein.
- **Ab-6** (clone 5B10C, Oncogene) is a mouse monoclonal antibody against **MDM2** that recognizes an epitope between amino acids 383 and 491 of

human MDM2 protein.

- **Ab-2** (clone 2A10, Oncogene) is a mouse monoclonal antibody against **MDM2** that recognizes an epitope between amino acids 294 and 339 of human MDM2 protein.
- **N-20** (Santa Cruz Biotechnology, Inc.) is a rabbit polyclonal antibody against **MDM2** raised against a peptide mapping at the N-terminal of human MDM2.
- **C-18** (Santa Cruz Biotechnology, Inc.) is a rabbit polyclonal antibody against **MDM2** raised against a peptide mapping at the C-terminal of human MDM2.
- **SMP14** (Santa Cruz Biotechnology, Inc.) is a mouse monoclonal antibody against **MDM2** raised against amino acids 154 - 167 of human MDM2.
- **2433** (Abcam) is a rabbit polyclonal antibody against **p53**, raised against a peptide corresponding to internal sequence amino acids 277-296 of human p53.
- **16B12** (Clone HA.11, Covance Research Products) is a mouse monoclonal antibody against **hemagglutinin (HA) tag** that was raised against the twelve amino acid peptide CYPYDVPDYASL.
- **12CA5** (Roche) is a mouse monoclonal antibody against **hemagglutinin (HA) tag** that recognizes the HA peptide sequence YPYDVPDYA derived from the human influenza hemagglutinin protein.
- **E-18** (Santa Cruz Biotechnology, Inc.) is a goat polyclonal antibody against **dihydrofolate reductase (DHFR)** raised against a peptide mapping within an internal region of human DHFR protein.
- **C-2** (Santa Cruz Biotechnology, Inc.) is a mouse monoclonal antibody against **actin** that recognizes an epitope within the C-terminus of human

actin. This antibody was primarily used to assess the total amount of protein loaded onto SDS-PAGE gel in western blotting.

- **Ab-1** (clone 200-193, Oncogene) is a mouse monoclonal antibody against **β-galactosidase** that recognises bacterial β-galactosidase and fusion proteins that contain the amino terminal 1020 amino acids of β-galactosidase. This antibody was used to assess the efficiency of transfection of mammalian cells by western blotting.
- **Leu-16** (clone L27, Becton Dickinson) is a mouse monoclonal antibody against **CD20** used as an immunoglobulin isotype control in immunoprecipitation experiments.

Unless stated otherwise, primary antibodies were used at the concentration of 3 μg /ml for detection of proteins by western blotting.

Secondary antibodies

- **Anti-mouse IgG** (Amersham/GE Healthcare Life Sciences) is a Horseradish Peroxidase (HRP) Linked Species-Specific Whole Antibody (from sheep) used at a dilution of 1:2500.
- **Anti-goat IgG** (Jackson Immunoresearch Laboratories Inc.) is a HRP-conjugated AffiniPure Mouse Anti-Goat IgG (H+L), used at a dilution of 1:20000.
- **Anti-rabbit IgG** (Amersham/GE Healthcare Life Sciences) is a Horseradish Peroxidase (HRP) Linked Species-Specific Whole Antibody (from donkey) used at a dilution of 1:5000.

2.3 Cell lines

H1299 (human non – small lung carcinoma cell line, p53 – null) and Clone 9 of H1299 cells (H1299-9) which overexpresses MDM2 are grown routinely in our laboratory. Cells were maintained in RPMI 1640 (Sigma) medium supplemented with 10% fetal bovine serum (FBS) (Sigma) at 37°C in a 95% air, 5% CO_2, humidified cell incubator.

HEK (human embryonic kidney) 293 cells and HEK 293 T cells (express the simian virus 40 (SV-40) Large T antigen) were maintained in DMEM HEPES modification medium supplemented with 10% FBS and 2 mmol/L L-glutamine (Sigma-Aldrich) at 37°C in a 95% air, 5% CO_2 , humidified cell incubator.

The High Five™ cell line (BTI – TN – 5B1 – 4) is derived from the *Trichoplusia ni* (cabbage looper) ovarian cells. Cells were grown at 27°C in the incubator, CO_2 exchange is not required for insect cell culture. Cells were maintained in EX – Cell 405 insect serum free medium (Sigma).

All cells were cultured as a monolayer using standard tissue culture techniques. Insect cells were also grown in suspension culture, prior and during virus infection.

2.3.1 Cell cultures techniques

All the work related to maintaining eukaryotic cells was performed in the laminar flow cabinet under sterile condition.

Mammalian cells were maintained in the media described in Cell line section and passaged upon reaching 90 – 100% confluence. In the first step, after discarding the growing medium, Dulbecco's phosphate buffered salin (PBS-Dulbeccos) (Sigma) was added to cells to wash them and remove any traces of serum, which is present in the growing media and capable of inhibiting Trypsin-EDTA activity. Following the washing step, PBS was discarded and Trypsin-EDTA (Sigma) solution was added and incubated for few minutes at 37°C to allow cell detachment from the flask surface. After short incubation, equal volume of growing media was added to neutralize trypsin and then according to future application, the required volume of cells mixture was added to a new flask containing fresh growing media.

Insect cells were maintained in the medium described in Cell line section and passaged upon reaching 80 - 90% confluence. Growing medium was discarded and 8 ml of fresh media added to the flask. In order to detach cells from flask surface, flask was harshly tapped few times. Upon cells detachment, 1ml of cell suspension was transferred to new flask filled with 19 ml of fresh growing medium.

2.3.2 Preparation of cryostocks

Buffers and reagents used

- Dimethyl sulfoxide (DMSO) (Sigma)
- Fetal bovine serum (FBS) (Sigma)
- EX – Cell 405 insect serum free medium (Sigma)

Mammalian cells were harvested by trypsinization and centrifuged for 5 minutes at 1000g. Supernatant was discarded and pellet resuspended in a solution of 10% DMSO in FBS to obtain cell concentration of $2x10^6$. Afterwards, 1ml of that mixture was transferred into cryotubes (Fisher) and stored overnight at –80°C, followed by transfer into liquid nitrogen tank.

In order to start growing frozen mammalian cells, they need to be thawed at 37°C followed by resuspention in 24 ml of appropriate growing medium and transfer into appropriate flask.

Insect cells (High five™) were harvested at ~80% confluence by tapping the flask containing cells growing in a monolayer and centrifuged for 5 minutes at 1000g. Supernatant was discarded and pellet resuspended in a solution of 10% DMSO in EX – Cell 405 medium. Afterwards, 1ml of that mixture was transferred into cryotubes (Fisher) and stored overnight at –80°C, followed by transfer into liquid nitrogen tank.

In order to start growing frozen insect cells (High five™), they need to be rapidly thawed at 37°C, followed by resuspension in 30 ml of EX – Cell 405 medium and centrifugation for 5 minutes at 1000g. After discarding the supernatant, cell pellet was resuspended in 20 ml of EX – Cell 405 medium and transferred into flask.

2.4 Transfection of mammalian cells

Transfection refers to delivery of exogenous DNA into mammalian cells.

Buffers and reagents used

- GeneJuice ® Transfection Reagent (Novagen)
- RPMI 1640 medium (Sigma)

H1299 cells were transfected using GeneJuice with a ratio of GeneJuice to DNA at 3 µl to 1 µg respectively, according to the manufacturer's protocol. Cells were at about 50% confluence at the time of transfection. GeneJuice was added to serum free RPMI 1640 medium, vortexed and left for 5 minutes at room temperature. DNA was added to GeneJuice – serum free medium, mixed by tapping the tube and incubated for 15 minutes at room temperature. After incubation, GeneJuice - DNA mixture was added drop-wise to the cells.

2.5 siRNA transfection of mammalian cells

Buffers and reagents used:

- Lipofectamine ^{TM} 2000 (Invitrogen)
- Opti – MEM® I reduced - serum medium (Invitrogen)
- siRNAs (Dharmacon) for MDM2:
    - MDM2#1 (5'GCCACAAAUCUGAUAGUA)
    - scrambled siRNA (5'GGACGCAUCCUUCUUAAU)

siRNA transfection was performed on H1299 and H1299-9 cells using Lipofectamine ^{TM} 2000 according to the manufacturer's instructions.

For a typical transfection of cells in 10 cm dishes, cells were plated one day before transfection to reach confluency of 30 – 50% at the time of tranfection. For each sample oligomer – Lipofectamine™ 2000 complexes were prepared as follows: 250 pmol of siRNA was diluted in 800 µl Opti-MEM® I reduced - serum medium, mixed by vortexing and incubated for 5 min at room temperature. The Lipofectamine™ 2000, 16 µl per 10 cm dish, was also diluted in 800 µl Opti-MEM® I reduced - serum medium, mixed by vortexing and incubated for 5 min at room temperature. After that incubation time, diluted siRNA and Lipofectamine™ 2000 were combined, mixed gently and incubated for another 20 min at room temperature. siRNA - Lipofectamine™ 2000 complexes were then added drop-wise to each 10 cm dish containing cells and 6.4 ml of growth medium. Content of each dish was mixed by rocking the plate back and forth. The final concentration of siRNA was 40nM. Medium in plates was changed after 6 hours. Twenty four hours after transfection cells from each 10 cm dish were split into two dishes, and harvested by trypsynization after another 24 h.

2.6 Bradford assay

Estimation of protein concentration in cell lysates was performed using Bradford assay based on an absorbance shift in the dye - Coomasie Brilliant Blue G – 250 upon binding to proteins.

Buffers and reagents used

Stuart Linn Immunoprecipitation Buffer (SLIP buffer)

- 50mM HEPES (pH 7.5) (Calbiochem)
- 150mM Sodium chloride (NaCl) (VWR)
- 10% Glycerol (Sigma)
- 0.1% Triton X-100 (Sigma)

Protein sample buffer

- 0.25M Tris (pH 6.8) (Calbiochem)
- 8% Sodium dodecyl sulphate (SDS) (VWR)
- 40% Glycerol (Sigma)
- 4mg/ml Bromophenol Blue (Sigma)
- 1% β-Mercaptoethanol (Sigma)

- Aprotinin (A) (2 μg/ml) (Roche)
- Leupeptin (L) (0.5 μg/ml) (Roche)
- Pepstatin (P) (1 μg/ml) (Roche)
- Soybean Trypsin Inhibitor (STI) (100 μg/ml) (Roche)
- Phenylmethylsulphonyl fluoride (PMSF) (1mM) (Fluka)
- Protein assay dye reagent concentrate (Biorad)
- Bovine serum albumin (BSA) (Fluka)

In order to estimate protein concentration in samples cell pellets were lysed on ice in SLIP buffer supplemented with protease inhibitors: aprotinin, leupeptin, pepstatin, soybean trypsin inhibitor and phenylmethylsulphonyl fluoride at the concentrations stated above. After 10 minutes incubation lysates were centrifuged at 16000xg at 4°C for 10 minutes and then supernatants transferred to clean tubes kept on ice. From each tube 2 μl of supernatant was taken and added to 1 ml of protein assay dye

reagent (diluted 1:5 with water), followed by measurement of absorbance at 595nm in spectrophotometer. The spectrophotometer was previously calibrated using 2 µl of protein standards in 1 ml of protein assay dye reagent (diluted 1:5 with water). Protein standards were prepared by mixing BSA with 1 ml of SLIP buffer to obtain protein concentration of 20, 10, 5, 2.5, 1.25, 0.6, 0.3 mg/ml. Protein concentration in samples was estimated based on comparison with standard curve of BSA with known concentrations ranging from 0.3 to 20 mg/ml. Based on protein concentration estimated for samples, usually 50 µg of total protein in 20 µl volume of sample buffer was prepared for further analysis by SDS-PAGE followed by western blotting.

2.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a commonly used technique, which enables separation of proteins on the gel according to their molecular size.

Buffers and reagents used

Component	SDS-PAGE resolving gel 10%	SDS-PAGE stacking gel
H_20	4.8 ml	7.225 ml
40% acrylamide mix (VWR)	2.5 ml	1.275 ml
1.5M Tris (pH 8.8) (Calbiochem)	2.5 ml	-
1 M Tris (pH 6.8)	-	1.25 ml
10% SDS (VWR)	0.1 ml	0.1 ml
10% Ammonium peroxosulfate (BDH)	0.1 ml	0.1 ml
10% N,N,N',N'-tetramethylethylenediamine (TEMED)	0.008 ml	0.010 ml

Table 2.7.1 Components of SDS-PAGE gels.

Tris-Glycine electrophoresis buffer (running buffer)

- 25mM Tris (Calbiochem)
- 250mM Glycine (Fisher)
- 0.1% SDS (VWR)

- Prestained protein marker, broad range (7-175 kDa) (NEB)

SDS-PAGE was performed using Mini-PROTEAN®-3 system supplied by Bio-Rad. Amount of acrylamide, thus the percentage of resolving gel may vary depending on the size of proteins to be analyzed (list of components for 10% gel is presented in Table 2.7.1), while stacking gel is always prepared with the same amount of acrylamide mix. Resolving gel was poured into glass cassette sandwich and allowed to polymerize, followed by pouring of stacking gel on the top and placing comb to create wells. After polymerization of stacking gel comb was removed and glass cassette placed in the tank filled with running buffer. Protein samples were boiled for a few minutes to allow protein denaturation and loaded on the gel, alongside with pre-stained protein marker as a size standard. Typically, a gel was run in a tank filled with running buffer for approximately 1 hour at 200 V.

2.7.1 Brilliant Blue G staining of polyacrylamide gel

After performing SDS-PAGE it is possible to visualize protein bands being present on the gel by staining with Coomasie Brilliant Blue G dye. Moreover, the staining enables to estimate the amount of analyzed protein when compared to the known protein amount on the same gel.

Buffers and reagent used

- Fixing solution (Sigma)
- Brilliant Blue G Colloidal Concentrate (Sigma)

After running, the gel was taken out from the glass cassette and placed for 30 minutes in a fixing solution to fix proteins on the gel prior staining with Brilliant Blue G dye. Following this, gel was placed in the dye and incubated for few hours in order to visualize protein bands on the gel, then to minimize the background gel was washed few times with water.

2.7.2 Western blotting

Western blotting is a immuno-technique which allows to identify specific proteins resolved by SDS-PAGE and transferred onto membrane.

Buffers and reagents used

Transfer buffer

- 25mM Tris (Calbiochem)
- 192mM Glycine (Fisher)
- 20% Methanol (BDH)

PBS/Tween

- 0.065M Na_2HPO_4 (Sigma)
- 0.015M $NaH_2PO_4xH_2O$ (Sigma)
- 0.075 NaCl (VWR)
- 0.1% Tween 20 (Sigma)

- Ponceau S (Sigma)
- Non-fat dry milk (Bio-Rad)
- Western lightning chemiluminescence reagent plus (Perkin Elmer)

The Mini trans-blot® electrophoretic transfer system (Bio-Rad) was used to transfer proteins from gel onto membrane. After resolving the samples, gel was placed on the Hybond ECL nitrocellulose membrane (GE Healthcare) in the cassette between sponges and Whatman paper. Following this, the cassette along with an ice pack (used to provide cooling during the transfer) was put in the tank filled with transfer buffer and transfer run for 1 hour at 100V. Afterwards, to check whether protein transfer was equal, membrane was stained with Ponceau S for 1 minute followed by several washes in water. Stained membrane was cut according to the size of proteins of interest with reference to the protein size marker. Following this, the membrane was completely destained with PBS/Tween and put in a 5% solution of non-fat dry milk in PBS/Tween for at least one hour in order to block nonspecific antibody binding to the membrane. After blocking, specific primary antibody was applied on the membrane, usually at the concentration of 3 μg/ml in 5% non-fat dry milk/PBS/Tween, and incubated for 1 hour with agitation. Afterwards, membrane was washed 3 times for 10 minutes in PBS/Tween and secondary antibody conjugated to horseradish peroxidase (HRP) applied on the membrane (e.g. at the dilution of 1:2500 for anti-mouse antibody in 5% non-fat dry milk in PBS/Tween) for 1 hour incubation with agitation. The membrane was washed 3 times for 10 minutes in PBS/Tween after incubation with secondary antibody. To visualize presence of specific proteins on the membrane after probing with primary and secondary HRP-conjugated antibody chemiluminescence reagent was used (ECL reagent). Following 1 min incubation with Western Lightning Chemiluminescence Plus ECL reagent, the membrane was either put in a cassette with the film or a Kodak IM4000 image station was used to detect luminescence signal.

2.8 *In vitro* binding assay

In vitro binding assay is a method which allows identification of direct interaction between two proteins (one of which is usually radiolabeled).

In vitro binding assay was performed using 6xHis-tagged MDM2 bound to Ni-NTA (nickel- nitrilotriacetic acid) agarose beads and *in vitro* translated (IVT), radiolabeled DNA polymerase α (catalytic subunit), as shown in Table 2.8.1. As a negative control 6xHis-tagged 23kDa fragment of the mycobacterial heat shock protein CPN60.1 (kindly provided by Dr. Peter Tormay) was used.

6xHis – tagged proteins	***In vitro* translated pol α (p180)**
Mouse MDM2 full length	pol α full length
	pol α N-terminal half
	pol α C-terminal half
Human MDM2 N-terminal half	pol α full length
	pol α N-terminal half
	pol α C-terminal half
Human MDM2 C-terminal half	pol α full length
	pol α N-terminal half
	pol α C-terminal half
Mouse MDM2 Δ166	pol α full length
	pol α N-terminal half
	pol α C-terminal half
CPN60.1	pol α full length
	pol α N-terminal half
	pol α C-terminal half

Table 2.8.1 *In vitro* binding assay pairings tested.

2.8.1 Protein preparation for *in vitro* binding assay

2.8.1.1 Purification of 6xHis–tagged MDM2

Buffers and reagents used:

Buffer B, pH 8.0

- 8 M Urea (BDH)
- 100mM NaH_2PO_4 (Sigma)
- 10mM Tris (Calbiochem)

Buffer C, pH 6.3 and 6.8

- 8 M Urea (BDH)
- 100mM NaH_2PO_4 (Sigma)
- 10mM Tris (Calbiochem)

Buffer D, pH 5.9 and 6.4

- 8 M Urea (BDH)
- 100mM NaH_2PO_4 (Sigma)
- 10mM Tris (Calbiochem)

Buffer E, pH 4.5 and 5.0

- 8 M Urea (BDH)
- 100mM NaH_2PO_4 (Sigma)
- 10mM Tris (Calbiochem)

Protein sample buffer

- 0.25M Tris (pH 6.8) (Calbiochem)
- 8% Sodium dodecyl sulphate (SDS) (VWR)
- 40% Glycerol (Sigma)
- 4mg/ml Bromophenol Blue (Sigma)
- 1% β-Mercaptoethanol (Sigma)

- Luria Bertani (LB) (Sigma)
- Ampicillin sodium salt (Sigma)
- Isopropyl – B – D –thiogalactopyranoside (IPTG) (VWR)
- L - Arabinose (VWR)
- Nickel-nitrilotriacetic acid (Ni – NTA) agarose beads (Qiagen)

Preparation of 6xHis–tagged proteins was performed according to QIAGEN manufacturer's The QIAexpressionist™ handbook.

Optimization of purification conditions

Prior to large scale purification, described below, small scale purification was performed in order to establish optimal conditions in terms of expression time course and also the pH value of buffers used.

1) Determination of optimal induction time for 6xHis-tagged MDM2

3 ml of Luria Bertani (LB) medium (containing appropriate antibiotic) was inoculated with 3 µl of glycerol stocks of bacterial cells harbouring expression construct (with gene of interest) introduced by transformation. Cultures were grown overnight at 37°C, with shaking at 225 rpm in bacteria incubator. Next day, 1 ml from the night culture was inoculated into 9 ml of LB and grown in the incubator until the culture has reached an OD600 (optical density at 600 nm) of 0.7 – 0.9. At this point, 1 ml of that bacterial culture was taken to serve as a non-induced control, followed by centrifugation at 15000g for 5 minutes, resuspension in 200 µl of buffer B and finally addition of 66 µl of 4x sample buffer. In order to induce protein expression, IPTG was added to the remaining bacterial culture to a final

concentration of 2mM. Sample volume of 1ml was collected 1, 2, 4, 6 and 8 hours after the addition of IPTG and processed as described for non-induced control. All samples were analyzed by SDS – PAGE followed by western blotting.

2) Optimization of wash buffer pH value for purification of 6xHis-tagged MDM2

Cultures were grown and protein expression induced as described above. Post induction cultures were incubated for 4 hours at 37°C, with shaking at 225 rpm in a bacteria incubator. Afterwards, cells were harvested by centrifugation at 4°C for 4 minutes at 3800g, pellets were resuspended in 2 ml of buffer B and lysates were then boiled for around 7 minutes to ensure proper cell lysis. In order to remove cellular debris lysates were centrifuged for 6 minutes at 3800 g. Afterwards, supernatants were transferred into fresh tubes and - 500 µl of a 50% Ni – NTA agarose beads slurry was added to each tube and incubated for 2 hours on a nutator at 4°C. After incubation the whole content of the tube was applied to Poly – Prep Chromatography Columns (Bio – Rad) and flow through was collected. In addition, to make sure that all of the beads were collected, walls of the tubes were washed with 2ml of buffer B and applied to the Bio - Rad columns. Columns were then washed with 5 x 500 µl of each of the buffers with two different values of pH as follows: buffer C pH 6.3 and 6.8, buffer D pH 5.9 and 6.4, buffer E 4.5 and 5.0 and each fraction (~500 µl) was collected. In order to test the amount of protein left on the beads after wash/elution with buffer E - 30 µl of beads was taken from each column and resuspended in 60 µl of 1x sample buffer. Each fraction was subject to SDS-PAGE and western blot analysis.

Large scale 6xHis-tagged protein purification

100 µl of the glycerol stocks of bacterial cells harbouring expression construct (with gene of interest) introduced by transformation was inoculated into 100 ml of autoclaved LB media containing appropriate antibiotic and this culture was grown overnight at 37°C shaking in the incubator. The next day 100ml of the overnight culture was inoculated into 900 ml of autoclaved LB media containing antibiotic, grown at 37°C in the incubator with shaking until OD600 was between 0.7 – 0.9 and to induce protein expression, IPTG was added to a final concentration of 2mM to MDM2 cultures. CPN60.1 expression was induced by adding L - arabinose to a final concentration of 0.04%. After 4 hours incubation cells from each culture were harvested by centrifugation for 5 minutes, at 3000 rpm at 4° C. After centrifugation supernatant was discarded and pellets stored at – 80° C overnight. The next day pellets were resuspended in 80 ml of buffer B (80 ml of buffer B per one day culture), lysates were transferred into 50 ml Corning tubes and boiled for 5 – 10 min. After cooling down for a few minutes, lysates were transferred into tubes suitable for centrifugation in Sorvall SS – 34 rotor and centrifuged for 30 minutes at 15000 rpm at 15° C. Supernatants were transferred into fresh 50 ml Corning tubes and Ni – NTA agarose beads were added (1 ml of 50% slurry per day culture) and incubated for 1 hour at 4° C on the nutator. After incubation tubes were placed at room temperature to allow settling down of the beads (for approximately 1 hour). Supernatants were removed (without disturbing beads pellets) and beads were resuspended in 10 ml of buffer B and again left for 1 hour to allow for settling down of the beads. After this supernatants were removed, beads resuspended in 10 ml of buffer B and applied to the Poly – Prep Chromatography Columns that were pre –

washed with H_2O. To make sure that all of the beads were collected, walls of the tubes were washed with 10 ml of buffer B and this was also applied to the column. Columns were then washed with 10 ml of buffer B, 15 ml of buffer C (pH 6.8) and 15 ml of buffer D (pH 6.4). Based on results from the small scale test purifications we determined that majority of the protein come off in washes with buffer E. Therefore, in order to have protein bound to the beads, washes were stopped after buffer D, columns were blocked, beads resuspended in buffer B (≈0.5 ml per column, to obtain 50% slurry) and stored at 4° C for future use.

2.8.1.2 *In vitro* transcription/translation of proteins

Buffers and reagents used:

- TNT® T7 Coupled Reticulocyte Lysate System (Promega) (see Table 4)
- [^{35}S] methionine (TRAN35S-Label™ ; MP Biomedicals, Inc.)
- RNasin® Ribonuclease Inhibitor (Promega)

In vitro protein synthesis was performed using TNT® T7 Coupled Reticulocyte Lysate System (Promega), which is a cell free system allowing for coupled transcripition/translation using phage T7 RNA polymerase and DNA template with the gene of interest cloned downstream from T7 RNA polymerase promoter e.g. in pSK-BBV vector (described in Plasmids and constructs section). Example of a typical TNT® T7 Coupled Reticulocyte Lysate System reaction using [^{35}S] methionine, according to Promega Technical Bulletin Part# TB126, has been presented below (Table 2.8.2).

Component	Volume
TNT® Rabbit Reticulocyte Lysate	25 µl
TNT® Reaction Buffer	2 µl
TNT® T7 RNA Polymerase	1 µl
Amino Acid Mixture, Minus Methionine (1mM)	1 µl
[^{35}S] Methionine (10mCi/ml)	4 µl
RNasin® Ribonuclease Inhibitor (40 U/µl)	1 µl
DNA template (1 µg) (gene of interest cloned into pSK-BBV vector)	
Nuclease-free H_2O	
Final volume	50 µl

Table 2.8.2 Components of *in vitro* transcription/translation reaction TNT® T7 Coupled Reticulocyte Lysate System (Promega). RNasin® Ribonuclease Inhibitor was supplied separately in order to create a ribonuclease – free environment; [^{35}S] methionine was added to the reaction to allow subsequent radioactive detection of *in vitro* synthesized protein.

The reaction mixture of the final volume of 50 µl was incubated at 30°C for 90 minutes, after incubation when needed stored in - 80 °C for the future use.

2.8.2 *In vitro* binding assay

Buffers and reagents used:

Buffer B, pH 8.0

- 8 M Urea (BDH)
- 100mM NaH_2PO_4 (Sigma)
- 10mM Tris (Calbiochem)

Dignam buffer

- 20mM HEPES (Calbiochem)
- 20% Glycerol (Sigma)
- 0.1M Potassium chloride (KCl) (VWR)

Protein sample buffer

- 0.25M Tris (pH 6.8) (Calbiochem)
- 8% SDS (VWR)
- 40% Glycerol (Sigma)
- 4mg/ml Bromophenol Blue (Sigma)
- 1% β-Mercaptoethanol (Sigma)

Fixing solution

H_2O: isopropanol: glacial acetic acid (65:25:10) (VWR)

- Aprotinin (A) (2 μg/ml) (Roche)
- Leupeptin (L) (0.5 μg/ml) (Roche)
- Pepstatin (P) (1 μg/ml) (Roche)
- Soybean Trypsin Inhibitor (STI) (100 μg/ml) (Roche)
- Phenylmethylsulphonyl fluoride (PMSF) (0.5mM) (Fluka)
- DL dithiothreitol (DTT) (0.5mM) (Sigma)
- Imidazole (VWR)
- Amplify™ Fluorographic Reagent (Amersham)
- Kodak GBX Developer and Fixer (Sigma)

In order to perform *in vitro* binding assay purified 6xHis–tagged proteins bound to Ni-NTA agarose beads and *in vitro* synthesized radiolabeled proteins were mixed as shown in Table 2.8.1.

Prior to starting the *in vitro* binding assay incubation, 6xHis-tagged proteins bound to the Ni-NTA beads which were kept in denaturated form in 8M urea buffer B, were renatured using serial dilution of 8M buffer B in Dignam buffer (starting with 4M and finishing with 0.06M) in the presence of protease inhibitors aprotonin, pepstatin, leupeptin, soybean trypsin inhibitor, phenylmethylsulphonyl fluoride and DL dithiothreitol. After washes with buffer B in Dignam buffer, 6xHis-tagged proteins bound to Ni-NTA beads were resuspended in Dignam buffer with protease inhibitors

to obtain 50% beads slurry - ready to use in the assay.

Afterwards, 100 µl of 50% slurry of 6xHis – tagged proteins bound to Ni-NTA beads was mixed with 10 µl of *in vitro* translated, radiolabeled proteins in 5 ml propylene tubes and incubated at 30° C for three hours, with shaking at 220 rpm. After incubation, contents of the tubes were transferred to 1.5 ml Eppendorf tubes and washed 4 times with 0.5 ml Dignam buffer (containing PMSF and DTT) supplemented with 75 mM imidazole. Beads were then resuspended in 30 µl of 1x sample buffer and run on the polyacrylamide gel, followed by gel fixing in H_2O: isopropanol: glacial acetic acid (65:25:10) solution for 30 minutes on shaking platform. Afterwards, gel was incubated with Amplify™ Fluorographic Reagent for another 30 minutes on a shaking platform followed by drying for 1 hour at 60° C (flat cycle; Gel dryer model 543, BIORAD). The dried gel was exposed to X – ray film (FujiFilm RX NIF sheets 180mm x 240mm (Fisher)) in a cassette at –80°C and developed using Kodak GBX Developer and Fixer in the darkroom.

2.9 p180 production and purification from the insect cells

Buffers and reagents used:

SLIP Buffer

- 50mM HEPES (pH 7.5) (Calbiochem)
- 150mM Sodium chloride (NaCl) (VWR)
- 10% Glycerol (Sigma)
- 0.1% Triton X-100 (Sigma)

- EX – cell 405 Insect Serum Free Medium (Sigma)
- Aprotinin (A) (2 µg/ml) (Roche)

- Leupeptin (L) (0.5 μg/ml) (Roche)
- Pepstatin (P) (1 μg/ml) (Roche)
- Soybean Trypsin Inhibitor (STI) (100 μg/ml) (Roche)
- β – mercaptoethanol (Sigma)
- Ni – NTA agarose beads (Qiagen)
- Imidazole (VWR)
- Brilliant Blue G-colloidal concentrate (Sigma)

Three 60 ml High Five cells suspension cultures (in 250ml flasks) were infected with pBlueBacHis2p180 (multiplicity of infection (MOI) 1 or conferring 1) at $1x10^6$ cells per milliliter cell density and incubated with a spin rate of 180 rpm for 96 hours at 27°C. Following this, cells were harvested by centrifugation and pellets resuspended in 30 ml of SLIP buffer, containing protease inhibitors (A, L, P, STI) and 20 mM of β – mercaptoethanol. After 30 minutes lysis on ice, lystes were clarified by centrifugation at 4° C for 15 minutes at 10500 rpm in Sorvall SS – 34 rotor. Supernatants were transferred into fresh tubes and 2 ml of 50% Ni – NTA slurry was added (prior use beads were washed 3 times with SLIP buffer). Supernatant with Ni-NTA beads was incubated for 90 minutes at 4°C on the nutator. Afterwards, mixture was passed through the Poly – Prep Chromatography Column (Bio-Rad) and washed with SLIP buffer supplemented with protease inhibitors and 20mM imidazole in order to remove unspecifically bound proteins. The column washes fractions were collected and protein presence monitored by reading optical density values at 280 nm in ultraviolet spectrophotometer; washing was continued until OD280 value was almost zero. Finally, DNA polymerase α was gradually eluted with 1ml of SLIP buffer (protease inhibitors included) containing 50mM, 100mM, 150mM and 200mM of imidazole. Aliquots from each fraction were taken

and analyzed by SDS-PAGE followed by Brilliant Blue G staining and western blotting.

2.10 Co-immunoprecipitation experiment

Co-immunoprecipitation is a technique used to identify proteins that exist in a complex within a cell, without discrimination between direct and indirect interaction. By using a specific antibody against the protein of interest it is possible to immunoprecipitate complexes formed by proteins *in vivo* and identify other binding partners forming the complex.

Buffers and reagents used:

SLIP buffer

- 50mM HEPES (pH 7.5) (Calbiochem)
- 150mM NaCl (VWR)
- 10% Glycerol (Sigma)
- 0.1% Triton X-100 (Sigma)
- 0.5mg/ml Bovine serum albumine (BSA) (Fluka)

Protein sample buffer

- 0.25M Tris (pH 6.8) (Calbiochem)
- 8% SDS (VWR)
- 40% Glycerol (Sigma)
- 4mg/ml Bromophenol Blue (Sigma)
- 1% β-Mercaptoethanol (Sigma)

- Aprotinin (2 μg/ml) (Roche)
- Leupeptin (0.5 μg/ml) (Roche)
- Pepstatin (1 μg/ml) (Roche)

- Soybean Trypsin Inhibitor (100 μg/ml) (Roche)
- Phenylmethanesulphonyl fluoride (PMSF) (1mM) (Fluka)
- Protein G sepharose beads (Sigma)

Antibodies

- anti - DNA polymerase α (N-19) (2 μg)
- anti - HA (16B12) (2 μg)
- anti - DHFR (E-18) (2 μg)
- anti - DNA polymerase α (HP180-12) (300 μl)
- anti - DNA polymerase α (SJK132-20) (300 μl)
- anti - CD20 (Leu16) (2 μg)

Cells were harvested 24 hours after transfection and resuspended in appropriate volume of SLIP buffer supplemented with protease inhibitors, lysed on ice for 10 minutes. Lysates were clarified by centrifugation at 14000 rpm at 4° C for 10 minutes and supernatants were transferred to a fresh tube. Protein concentration in the supernatants was measured using Bradford Assay, based on which 4 mg of each sample was taken to be used for each immunoprecipitation reaction. In order to minimize the amount of proteins that bind nonspecifically to Protein G beads, samples were gently mixed on the Nutator with 50 μl of 50% slurry of Protein G sepharose beads for 1 hour at 4°C. Afterwards, samples were briefly centrifuged and pre-cleared supernatants transferred into fresh tubes, followed by addition of 2 μg of either specific antibody or isotype control and incubation for 2 hours at 4°C with gentle mixing on a nutator. In the next step 50 μl of Protein G 50% slurry was added and samples were incubated for another 2 hours under the same conditions, to capture the immuno complex on Protein G beads. Afterwards, Protein G beads were washed three times with 0.5 ml of SLIP buffer containing protease inhibitors,

resuspended in 40 µl of 1x protein samples buffer and stored at -80 °C for future analysis by SDS-PAGE and western blotting.

2.11 Purification of GST-MDM2 and GST

Glutathione S-transferase (GST), a 26kDa enzyme with a high affinity to glutathione, is commonly used as a protein tag. Expression and purification of GST fusion proteins from bacterial cells allows to obtain large quantities of relatively pure protein with preserved function since protein purification/elution is performed under mild conditions.

Buffers and reagents used:

SLIP Buffer

- 50mM HEPES (pH 7.5) (Calbiochem)
- 150mM NaCl (VWR)
- 10% Glycerol (Sigma)
- 0.1% Triton X-100 (Sigma)

- Aprotinin (2 µg/ml) (Roche)
- Leupeptin (0.5 µg/ml) (Roche)
- Pepstatin (1 µg/ml) (Roche)
- Soybean Trypsin Inhibitor (100 µg/ml) (Roche)
- Phenylmethylsulphonyl fluoride (PMSF) (1mM) (Fluka)
- L-Glutathione (Sigma)
- Glutathione Sepharose 4B (GE Healthcare)
- Isopropyl – B – D –thiogalactopyranoside (IPTG) (VWR)
- Ampicillin sodium salt (Sigma)
- Luria Bertani (LB) (Sigma)

40 µl of pGEX2TK-GSTMdm2 glycerol stock (or pGEX-6P-1 GST in case of purification of GST) was inoculated into 100 ml of autoclaved LB media supplemented with ampicillin and grown overnight at 37°C, shaking (250rpm) in the incubator. The next day 100ml of the overnight culture was inoculated into 900 ml of autoclaved LB medium containing ampicillin and grown until OD600 was between 0.6 – 0.8. To induce protein expression, IPTG was added to a final concentration of 0.5mM and bacterial culture was grown for additional 4 hours at the same conditions as described above. Cells were then harvested by centrifugation for 5 minutes, at 8000 rpm at 4° C, supernatant was discarded and pellet stored at – 80° C overnight. The next day pellet was resuspended in 40 ml of SLIP buffer and lysed on ice for at least 20 minutes. The lysate was transferred into 50 ml Sorvall centrifuge tubes and sonicated three times for 30 seconds at power output of 25%, and then left on ice for at least 10 minutes. Following this, bacterial debris was pelleted by centrifugation for 10 minutes, at 15000g at 4° C, the clarified lysate was transferred into 50 ml Corning tube, glutathione beads were added to the lysate (0.5 ml of 50% slurry, washed three times with SLIP buffer) and incubated for 2 hours at 4° C on the nutator. After incubation the content of the tube was applied to Poly – Prep Chromatography Column and allowed to drip through. The column was washed two times with 15 ml of SLIP buffer. Finally, 1ml of SLIP buffer containing 20 mM of L-Glutathione was added and incubated for 10 minutes to elute protein from the column (beads). Afterwards, a small fraction of eluted GST tagged protein (or GST) was subjected to SDS-PAGE followed by Brilliant Blue G staining and western blotting in order to confirm presence of protein of interest in the eluate. Remaining fraction of eluted protein was used in DNA polymerase assay.

2.12 *In vitro* ubiquitylation assay with GST-MDM2

MDM2 mediates ubiquitylation of many of its interacting partners, as well as its own ubiquitylation due to its E3 ubiquitin ligase activity (64). This enzymatic activity of MDM2 can be tested *in vitro* by performing ubiquitylation assay, using GST-MDM2 purified under non-denaturing conditions.

Buffers and reagents used

Reaction buffer 10x	**Volume (µl)**
Tris HCl (1M pH 7.5)	50
$MgCl_2$ (250mM)	2
ATP (80mM)	25
DTT (1M)	2
$ZnCl_2$ (10mM)	2
H_2O	19
Final volume	100

Table 2.12.1 Components of *in vitro* ubiquitylation assay reaction buffer.

SLIP Buffer

- 50mM HEPES (pH 7.5) (Calbiochem)
- 150mM Sodium chloride (NaCl) (VWR)
- 10% Glycerol (Sigma)
- 0.1% Triton X-100 (Sigma)

- Ubiquitin activating enzyme E1 (UBE1) (Boston Biochem)
- Ubiquitin conjugating enzyme E2 (UbcH5B) (Boston Biochem)
- Ubiquitin (Boston Biochem)

In vitro ubiquitylation assay should be performed on the same day as purification of GST-MDM2, used in the assay, in order to retain the E3 ligase activity of MDM2.

Ubiquitylation reactions were set up in 1.5ml Eppendorf tubes in a volume of 100 µl in the following order:

1) SLIP buffer
2) 10 x reaction buffer
3) substrate protein (this component is omitted when testing autoubiquitylation of MDM2)
4) GST-MDM2 or GST (negative control) (approximately 200ng)
5) Either "ubiquitin mix" consisting of 100ng of E1, 400ng of E2, and 10 µg of ubiquitin or "no ubiquitin" control consisting of 100ng of E1, 400ng of E2, and water instead of ubiquitin.

Reactions were incubated at 30°C overnight and stopped by adding sample buffer and samples were subjected to SDS-PAGE followed by western blotting.

2.13 DNA polymerase assay

The DNA polymerase assay is used to measure of the enzyme activity, based on the incorporation of radiolabeled nucleotides e.g. ^{3}H dTTP. It is possible to test whether addition of other proteins has any effect on DNA polymerase enzymatic activity using this assay.

Buffers and reagents used

Reaction buffer	**Add** (µl)	**Final concentration**
^{3}H dTTP (5.8 pmol/µl)	33	1.91 pmol/µl
dTTP (10 mM)	0.2	20 µM
Poly (dA) oligo (dT) (1µg/µl)	5	50 µM
DTT (0.5M)	2	10mM
$MgCl_2$ (1M)	1.5	15mM
HEPES-KOH pH 7.5 (1M)	5	50mM
BSA (10mg/ml)	2	0.2 mg/ml
Glycerol (50%)	40	20%
Triton x-100 (1%)	3	0.03%
H_2O	8.3	
Final volume	100 µl	

Table 2.13.1 Components of reaction buffer for DNA polymerase assay.

Assay buffer

- 50 mM HEPES-KOH (pH 7.5) (Calbiochem)
- 10 mM DL dithiothreitol (DTT) (Sigma)
- 15 mM Magnesium chloride ($MgCl_2$) (VWR)
- 10% Glycerol (Sigma)
- 0.03% Triton-X 100 (Sigma)

STOP solution

- 10% Trichloroacetic acid (TCA) (BDH)
- 0.05M Sodium pyrophosphate tetrabasic decahydrate ($Na_4P_2O_7$) (Sigma)

Washing buffer

- 1M Hydrochloric acid (HCl) (VWR)
- 0.1M Sodium pyrophosphate tetrabasic decahydrate ($Na_4P_2O_7$) (Sigma)

- OSC Scintillation Cocktail (Amersham)

A typical DNA polymerase assay reaction mix is assembled on ice and contains:

1) enzyme e.g. DNA polymerase α
2) protein of interest e.g. GST-MDM2 or control protein e.g. GST
3) assay buffer

} total volume of 10 µl

4) 90 µl of reaction buffer (components presented in Table 2.13.1)

Reaction mix (in quadruplicate per each condition) was incubated for one hour at 37°C in a sealed 96-well plate. Afterwards, the plate was put on ice and content of each well transferred into a 5 ml tube, followed by addition of 500 µl of STOP solution terminate the reaction. Content of each tube was transferred onto glass microfibre filter (Whatman GF/A 25mm (Fisher)) to collect acid - precipitable (radioactive) products of reaction and the filter was then washed with ~20 ml of washing buffer. The next day filters were put into the scintillation vials and after addition of 5 ml OSC Scintillation Cocktail (to the vials) radioactivity was counted using a Packard 1500 Tri-Carb liquid scintillation analyzer.

2.14 *In vivo* ubiquitylation/NEDDylation assay

In vivo ubiquitylation (/NEDDylation) assay is used for identification of substrates for ubiquitin (/NEDD8) in the cell. This technique is based on usage of Ni-NTA agarose beads to pull down the 6xHis-tagged ubiquitin (/NEDD8) covalently attached to its protein substrates.

Buffers and reagents used

SLIP Buffer

- 50mM HEPES (pH 7.5) (Calbiochem)
- 150mM NaCl (VWR)
- 10% Glycerol (Sigma)
- 0.1% Triton X-100 (Sigma)
- 0.5mg/ml Bovine Serum Albumine (BSA) (Fluka)

Buffer A (pH 8.0)

- 6M Guanidine hydrochloride (Sigma)
- 0.1M NaH_2PO_4 (Sigma)
- 0.01M Tris (Calbiochem)
- 5mM Imidazole (VWR)
- 10mM β-mercaptoethanol (Sigma)

Buffer B (pH 8.0)

- 8M Urea (BDH)
- 0.1M NaH_2PO_4 (Sigma)
- 0.01M Tris (Calbiochem)
- 10mM β-mercaptoethanol (Sigma)

Buffer C (pH 6.3)

- 8M Urea (BDH)
- 0.1M NaH_2PO_4 (Sigma)
- 0.01M Tris (Calbiochem)
- 10mM β-mercaptoethanol (Sigma)

Protein sample buffer

- 0.25M Tris (pH 6.8) (Calbiochem)
- 8% SDS (VWR)
- 40% Glycerol (Sigma)
- 4mg/ml Bromophenol Blue (Sigma)
- 1% β-Mercaptoethanol (Sigma)

- GeneJuice ® Transfection Reagent (Novagen)
- RPMI 1640 medium (Sigma)
- Aprotinin (2 μg/ml) (Roche)
- Leupeptin (0.5 μg/ml) (Roche)
- Pepstatin (1 μg/ml) (Roche)
- Soybean Trypsin Inhibitor (100 μg/ml) (Roche)
- Phenylmethylsulphonylfluoride (PMSF) (1mM) (Fluka)
- Ni – NTA agarose beads (Qiagen)
- Imidazole (VWR)
- 10mM *N*-Ethylmaleimide (NEM)

H1299 cells were transfected with 15 μg of His-Ubiquitin (or His-NEDD8 - pcDNA3 NEDD8 in case of NEDDylation assay) expression plasmid per 15cm dish in addition to other expression constructs using GeneJuice transfection reagent. Twenty four hours after transfection cells were split 1:2 and grown for an additional 24 hours after which cells were harvested into two aliquots: 10% for western blotting (to check for the presence and possible degradation of transfected proteins of interest) and 90% for the assay purpose (purification with Ni-NTA beads). In order to perform the purification process, cell pellets (90%) were lysed in 2 ml of buffer A (1ml per 15cm dish) supplemented with 10mM NEM in order to prevent deubiquitylation. 200 μl of Ni-NTA beads (50% slurry) was added to the lysates and samples were incubated for four hours on the nutator at room temperature. After incubation Ni-NTA beads were washed with 1 ml of buffer A, 1ml of buffer B and 1ml of buffer C in order to remove proteins unspecifically bound to the beads. Following washes, proteins bound to the Ni-NTA beads were eluted into 50 μl of 1 x sample buffer supplemented with 200mM imidazole and analyzed by SDS-PAGE followed by western blotting.

2.15 Flow cytometry analysis

Buffers and reagents used:

- Ethanol (ice cold) 70% (VWR)
- Phosphate buffered saline (PBS), PBS Tween 20 (0.1%) (Sigma)
- Ribonuclease A (RNase A) (10 mg/ml) (Sigma)
- Propidium iodide (PI) in PBS (1 mg/ml) (Sigma)

Typically, each sample prepared for analysis should contain between 0.5 – $1x10^6$ cells in total. Mammalian cells were grown and harvested by trypsinization followed by centrifugation for 5 minutes at 173g at room temperature. Cell pellets were then washed in PBS and centrifuged twice. After last centrifugation PBS was discarded and tubes containing the pellets were harshly flicked to resuspend the cells and create a single cell suspension. Cells were gently vortexed while adding drop-wise 5 ml of 70% ice cold ethanol. Samples were left on ice for at least 1 hour (or up to two weeks in the fridge). During the next step cells were pelleted by centrifugation and washed twice in PBS Tween 20, resuspending cells each time. After pelleting the cells for the final time, PBS Tween was removed, pellets resuspended in 100 µl of RNase A and left on ice for 5 minutes. Staining solution containing 850 µl of PBS and 50 µl of propidium iodide was added to each sample to be analyzed and left on ice in the dark for 30 minutes.

Samples were analyzed using a Beckman Coulter Epics XL flow cytometer using FL2 (propidium iodide) channel. Data were analyzed using WIN MDI software (Windows multiple document interface for flow cytometry; Scripps Research Institute) and Cylchred software (developed at the University of Cardiff).

2.16 Thymidine incorporation assay

Thymidine incorporation assay is used to measure cells proliferation rate based on incorporation of [^{3}H]-thymidine into nucleic acid.

Buffers and reagents used:

- RPMI 1640 medium (Sigma) + 10% fetal bovine serum (FBS) (Sigma)
- [^{3}H]-thymidine (1mCi/ml)
- Phosphate Buffered Saline (PBS) (Sigma)
- 5% Trichloroacetic acid (TCA) (BDH)
- 0.1M Sodium hydroxide (NaOH) (VWR)
- OSC Scintillation Cocktail (Amersham)

Cells were plated at 5 x 10^4 per well (6 well plate) in 2 ml growth medium and incubated for 24 hours in cell incubator at 37°C. [^{3}H]-thymidine was added to each well (apart from negative control wells) to final concentration of 1 μCi/ml and samples were incubated for 2 hours in cell incubator. After incubation medium containing [^{3}H]-thymidine was discarded and cells were washed three times with ice cold PBS. 5% TCA was added to each well and incubated for 20 minutes in the fridge, in order to precipitate radioactive products. The TCA was discarded and wells washed two times with ethanol. 1ml of 0.1M NaOH was added to each well and plates were put in the oven at 60°C for 1 hour. Following this 200 μl from each well was added to 5ml of OSC Scintillation Cocktail in scintillation vials, the vials were shaken well and radioactivity counted using a Packard 1500 Tri-Carb liquid scintillation analyzer.

The assay was performed in triplicate.

CHAPTER III

RESULTS

MDM2, in addition to its interplay with p53, interacts with several others proteins and many of these are involved in crucial cell processes (2). One examples, reported by members of our research group, appears to implicate MDM2 in some way in the process of DNA replication and/or repair via interaction with DNA polymerase ε (58,59). During the course of studies on MDM2 and DNA polymerase ε, it was also discovered that MDM2 interacts with DNA polymerase α.

Several experiments on MDM2 and DNA polymerase α interaction were carried out by the former members of our research group – Dr. Maria Maguire and Dr. Paul Nield prior to the commencement of studies described in this thesis. These experiments involved affinity chromatography based on which we concluded that DNA polymerase α co-purified with 6xHis-tagged MDM2 from a nickel-charged column (Figure 3.1.1). These findings were then additionally confirmed by the results from immunoprecipitation experiments on untransfected HEK293 cells which demonstrated that a protein complex exists containing both DNA polymerase α and MDM2 in these cells, as presented in Figure 3.2.1 of co-immunoprecipitation experiments section (3.2).

These studies suggest that complexes exists that contains both DNA polymerase α and MDM2 but did not address the important question of whether MDM2 and DNA polymerase α interact directly. To further examine the interaction of MDM2 with DNA polymerase α, we first performed *in vitro* binding assay since these

enables us to examine direct interactions between proteins. Next, we examined protein complexes by co-immunoprecipitation with MDM2 deletion mutants to determine which regions of MDM2 were required to incorporate MDM2 into the complex and finally examined whether MDM2 had any effect on the DNA polymerase activity of DNA polymerase α or whether due to its E3 ligase activity MDM2 mediated modifications of the catalytic subunit of DNA polymerase α (p180).

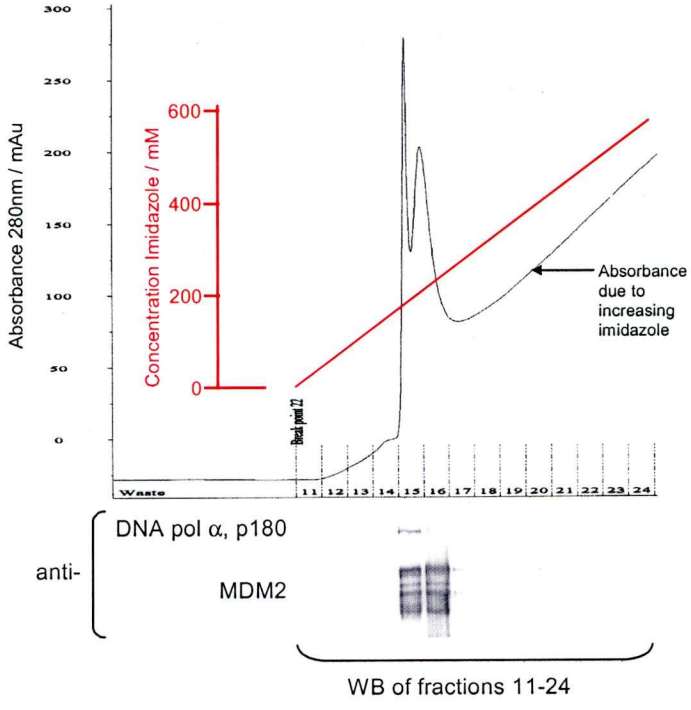


Figure 3.1.1 DNA polymerase α co-purifies with MDM2. Upper panel: elution profile from the MDM2 coulmn; 5mg of MDM2 was bound to a HiTrap column charged with Ni++ and then 12mg of BSA was applied to the column. After washing the column extensively 12mg total protein lysate from HEK293 cells was passed over the column and then again washed extensively. Ni++ binding proteins were then eluted with an imidazole gradient from 0-1M. Lower panel: western blot of fractions 11-24 probed with the anti-p180 (N-19) antibody for detection of DNA polymerase α and anti-MDM2 (Ab1, clone IF2) (experiment was performed by Dr. Maria Maguire and Dr. Paul Nield).

3.1 Investigation of direct interaction between MDM2 and DNA polymerase α

Investigation of the potential direct interaction between MDM2 and DNA polymerase α was performed using an *in vitro* binding assay. This method has been used before in our laboratory (56,58) and enables one to determine whether two proteins incorporated into the assay interact directly. For the assay one of the protein partners is usually radiolabeled, while the other one is a 6xHis-tagged protein bound to Ni-NTA beads. Therefore, in order to obtain radiolabeled proteins a TNT® T7 Coupled Reticulocyte Lysate System (Promega) with [^{35}S] methionine was used (as described in Materials and methods, section 2.8.1.2). For the 6xHis-tagged proteins, expression and purification was based on the QIAexpress® System (Qiagen), with a set of pilot experiments performed to optimize conditions for expression and purification of proteins of interest.

3.1.1 Expression and purification of 6xHis-tagged proteins

3.1.1.1 Determination of optimal induction time for 6xHis-tagged MDM2

As stated above, the QIAexpress® System (Qiagen) was used to produce 6xHis-tagged proteins in bacterial cells, since this allows us to obtain relatively high level expression of fusion proteins compared to many other expression systems e.g. insect or mammalian cells. In order to determine the optimal induction time for expression of proteins of interest, a pilot experiment was performed as described in Materials and methods section 2.8.1.1.

Time course experiments for 6xHis–tagged human MDM2, amino (N)-terminal half of MDM2 (246 amino acids) and carboxy (C)-terminal half MDM2 (252 amino acids) were carried out with sample collection at times 1, 2, 4, 6 and 8 hours after induction with IPTG. All samples were then subjected to SDS – PAGE analysis followed by western blot performed using anti-MDM2 antibodies: Ab1 (clone IF2) for MDM2 full length and amino (N)-terminal half MDM2, and Ab 6 (clone 5B10C) for carboxy (C)-terminal half MDM2. Expected molecular weights of all MDM2 constructs were as follows: MDM2 - ~ 90kDa, amino (N)-terminal half MDM2 - ~ 42 kDa, carboxy (C)-terminal half MDM2 - ~ 50 kDa. As shown in Figure 3.1.2 A and B, 3.1.2 A represents a shorter exposure and 3.1.2 B a longer exposure of the same blot, levels of expression at 4, 6 and 8 hours upon induction are comparable. Based on the signal strength displayed on blots (Figure 3.1.2), 4 hours induction with IPTG was chosen as optimal for the future experiments since it confers high protein expression levels but within a shorter period of time and this may result in less toxicity and protein degradation than might arise at later time points; 4 hours induction was also recommended as a starting point in the QIAexpressionist™ manual for the QIAexpress® System (Qiagen).

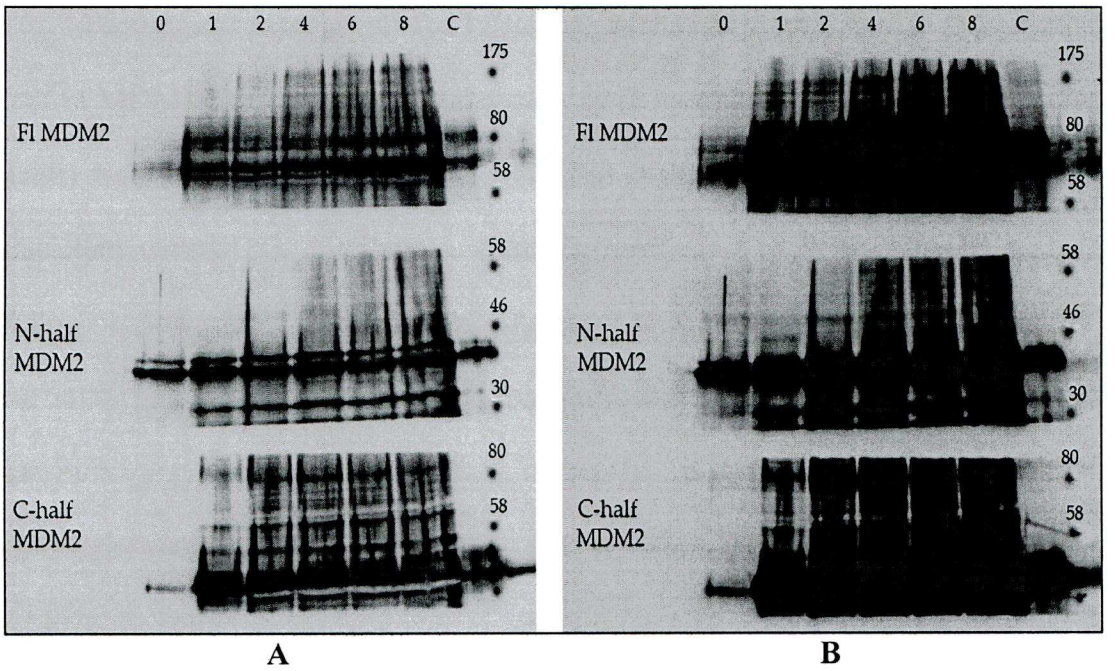


Figure 3.1.2 Test of the time of induction of expression of 6xHis-tagged MDM2 using IPTG. Western blot of full length MDM2 and its amino (N)- and carboxy (C)-terminal halves expressed in XL – 1 Blue cells, harvested 1, 2, 4, 6 and 8 hours after induction with IPTG. Time 0 also served as a noninduced control, C – purified protein - represents positive control for each protein. Equal volumes of analyzed samples represent 2% of the bacterial lysate; Ab1 (clone IF2) antibody was used for detection of full length and amino-terminal half of MDM2, while Ab-6 (clone 5B10C) antibody was used to detect carboxy-terminal half of MDM2 (**A** – short exposure, **B** – long exposure).

3.1.1.2 Optimization of wash buffer pH value for purification of 6xHis-tagged MDM2

Purification of 6xHis-tagged proteins can be performed either under native or denaturing conditions. The bacterial expression system used for this experiment ensures high expression of proteins of interest, which may however lead to the formation of insoluble protein aggregates. Hence, in order to solubilize these potential aggregates, purification under denaturing condition was chosen (previous

experience has shown that much lower levels of MDM2 are produced under native conditions). Moreover, purification under denaturing conditions guarantees improved binding of 6xHis-tagged protein to the Ni-NTA, since the tag is fully exposed, and also there is reduced nonspecific binding of untagged proteins. To ensure optimal purification of the protein of interest, other conditions need to be determined as well, and one particularly important is the pH of the 8M urea buffers used throughout the procedure. Urea buffer at pH 8.0 (buffer B) was used for bacterial cells lysis and subsequent binding of fusion proteins to Ni-NTA, whereas for washing (removal of nonspecifically bound proteins) and elution steps the buffer pH should be lower than 8.0 because at lower pH histidine (His) residues become protonated thus reducing the electrostatic attraction between histidine and nickel (Ni) ions.

In order to determine the optimal buffer pH for washing and elution steps, buffers with pH values of 6.3 (buffer C), 5.9 (buffer D) and 4.5 (buffer E) were initially tested during small scale protein purification, as suggested in the QIAexpressionist™ manual. As shown in figure 3.1.3 A and A', when buffers with pH 6.3 (C), 5.9 (D) and 4.5 (E) were applied to the column containing carboxy- and amino-terminal halves of MDM2 bound to Ni-NTA beads, the proteins of interest were eluted in almost every fraction, instead of being present only in fractions collected from washes with buffer E. To decrease the stringency of the washes, the pH of the buffers was increased to 6.8, 6.4 and 5.0 for C, D, and E buffers, respectively. This improved the elution profile of the proteins and resulted in the majority of recombinant protein eluting in fractions E1 and E2, as shown in Figures 3.1.3 B - B'.

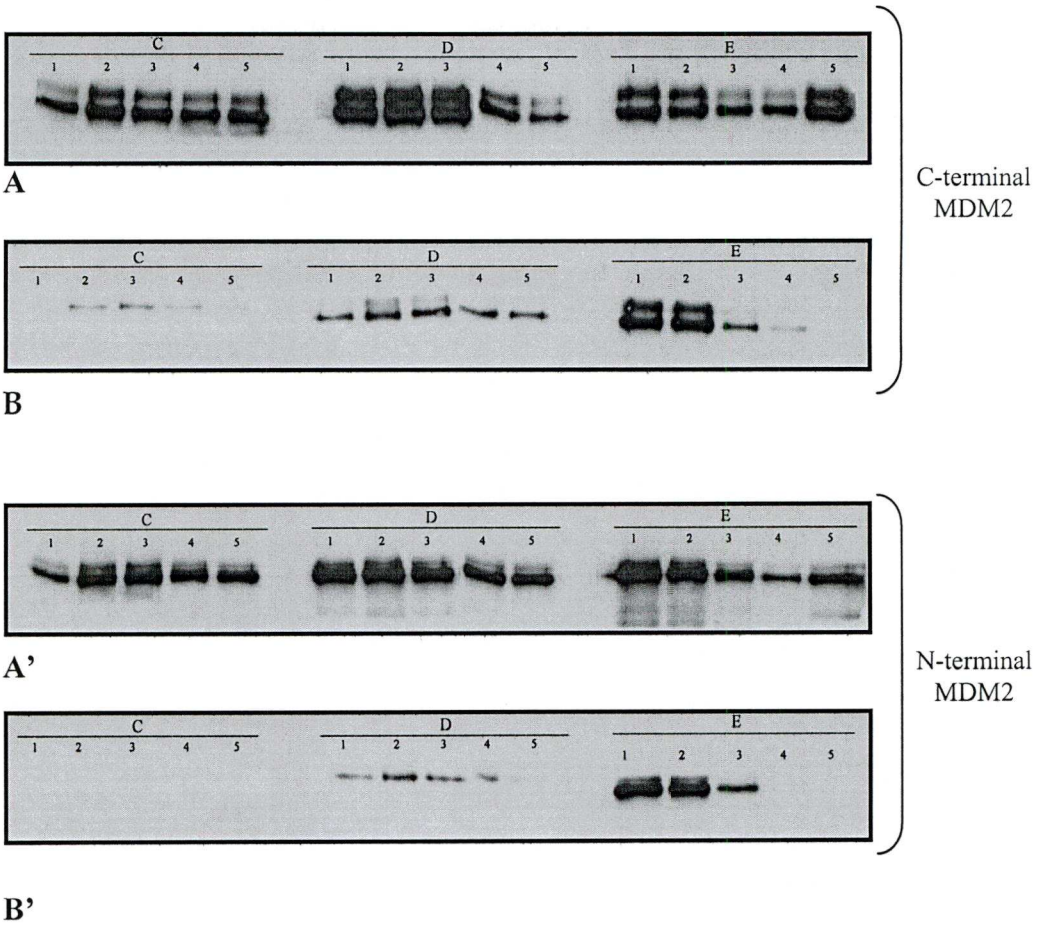


Figure 3.1.3 Optimization of buffer pH for purification of 6xHis-tagged proteins. Western blot of carboxy (C)- and amino (N)- terminal halves of human MDM2 expressed in XL – 1 Blue cells. Numbers from 1 to 5 represent five washes (0.5ml) with each buffer. **A and A')** buffer pH: C – 6.3, D – 5.9, E – 4.5; **B and B')** buffer pH: C – 6.8, D – 6.4, E – 5.0. Equal volumes of analyzed samples represent 4% of the bacterial lysate; Ab1 (clone IF2) antibody was used for detection of the amino-terminal half of MDM2, while Ab-6 (clone 5B10C) antibody was used to detect carboxy-terminal half of MDM2.

3.1.1.3 Large scale purification of 6xHis-tagged MDM2

The small scale pilot experiments (described in sections 3.1.1.1 and 3.1.1.2) allowed us to determine the optimal conditions for expression and purification of 6xHis-tagged MDM2 from bacterial cells (XL-1). Therefore, for large scale expression and purification of 6xHis-tagged MDM2 a 4 hour induction with IPTG was performed, followed by purification of fusion proteins on Ni-NTA beads using 8M urea buffer at pH 6.8 (buffer C) and 6.4 (buffer D). In order to retain protein bound to the beads the last wash – elution with buffer E was omitted. After washes with buffer C and D, a fraction of the protein left on the beads was subjected to 15% SDS – PAGE analysis, followed by Brilliant Blue G staining (Figure 3.1.4) and western blot analysis using appropriate antibodies (anti-MDM2: Ab 1, Ab 2 and Ab 6) (Figure 3.1.5) (expected molecular weights: MDM2 at ~ 90kDa, amino-terminal half MDM2 at ~ 42 kDa and carboxy-terminal half MDM2 at ~ 50 kDa). A typical purification result is shown in Figure 3.1.4 and it can be seen that the levels of MDM2/MDM2-fragment expression were variable. The highest amount of protein was present in the lane corresponding to the carboxy-terminal half of MDM2, while the expression levels of full length MDM2, the amino-terminal half of MDM2, and the control protein CPN60.1 are present at lower levels with the full length mouse MDM2 expressed at the lowest level. When the same fractions were analyzed by western blotting, this analysis confirmed the presence of MDM2 proteins on the beads (Figure 3.1.5).

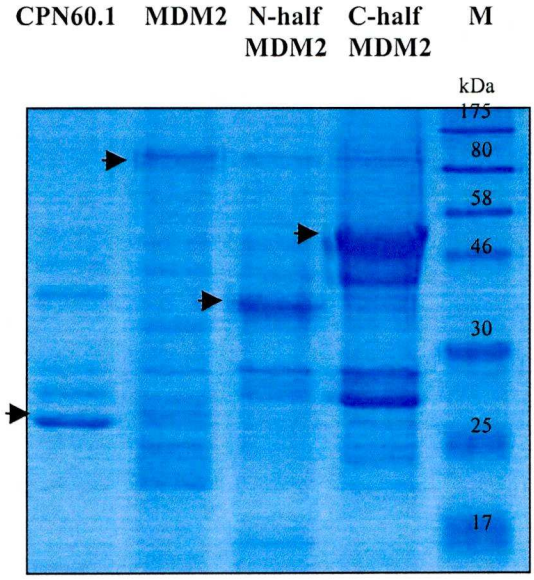


Figure 3.1.4 Brilliant Blue G staining of 6xHis-tagged CPN60.1, MDM2, amino- and carboxy-terminal halves of MDM2. 6xHis-tagged proteins were bound to the Ni-NTA beads after purification procedure and then separated on 15% polyacrylamide gel. Main protein bands are indicated by arrows: CPN60.1 at ~ 27 kDa, MDM2 at ~ 90kDa, amino (N)-terminal half MDM2 at ~ 42 kDa and carboxy (C)-terminal half MDM2 at ~ 50 kDa; M – indicates protein molecular weight marker.

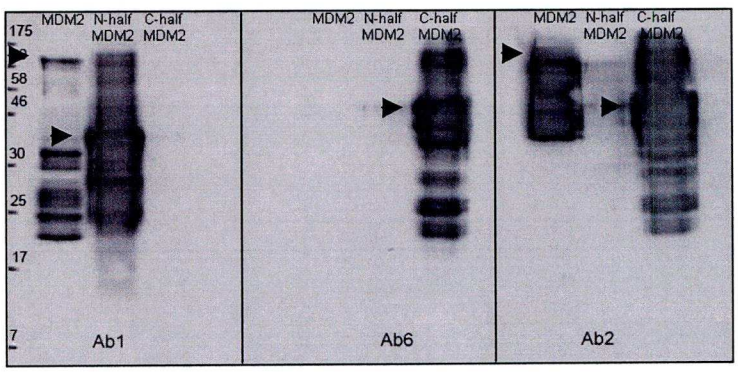


Figure 3.1.5 Western blot analysis of 6xHis–tagged MDM2 proteins bound to the Ni-NTA beads after purification procedure. Proteins were separated on 15% polyacrylamide gel. Equal volumes of analyzed samples represent 3% of the total protein bound to the Ni-NTA beads; Ab1, Ab6 and Ab2 antibodies were used for detection of MDM2 proteins as indicated at the bottom of the figure. Main protein bands are indicated by arrows: MDM2 at ~ 90kDa, amino (N)-terminal half MDM2 at ~ 42 kDa and carboxy (C)-terminal half MDM2 at ~ 50 kDa.

3.1.2 *In vitro* binding assay – 6xHis-tagged p53 protein binds to p180 *in vitro*

After establishing the conditions for purification of 6xHis-tagged MDM2 proteins (sections 3.1.1.1-3) we decided to perform a pilot *in vitro* binding assay utilising well known and acknowledged binding partners, in order to examine the specificity of the assay.

Since p53 is the best established and most widely studied MDM2 interacting protein, with a proven profound effect on cell function (section 1.4) it was an obvious candidate for the pilot experiment. The assay was carried out, as described in Materials and methods (section 2.8), with 6xHis-tagged p53 (Figure 3.1.6B) and CPN60.1 (Figure 3.1.6A) proteins purified by binding to the Ni-NTA beads and incubated with *in vitro* translated MDM2 protein for 3 hours at 30°C and then washed extensively to remove non-specific interactions. Samples were then analyzed by SDS-PAGE and fluorography. Results presented in Figure 3.1.7 show, as expected, that *in vitro* translated MDM2 binds directly to 6xHis-tagged p53 and does not bind to CPN60.1 protein.

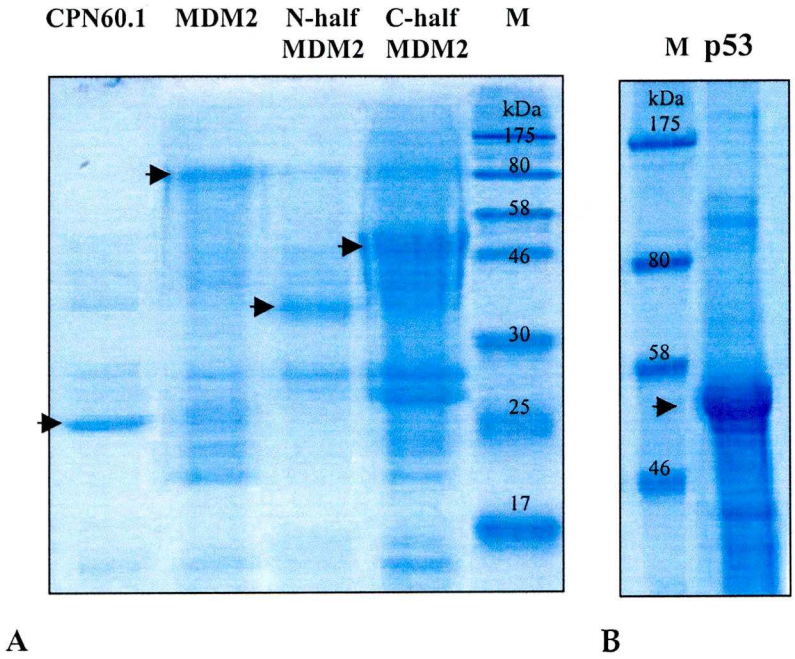


Figure 3.1.6 Brilliant Blue G staining of 6xHis-tagged CPN60.1, MDM2, amino- and carboxy-terminal halves of MDM2 and p53. Brilliant Blue G staining of 6xHis-tagged proteins bound to the Ni-NTA beads after purification procedure, separated on 15% polyacrylamide gel (**A**) and 10% polyacrylamide gel (**B**). Main protein bands are indicated by arrows: CPN60.1 at ~ 27 kDa, MDM2 at ~ 90kDa, amino (N)-terminal half MDM2 at ~ 42 kDa, carboxy (C)-terminal half MDM2 at ~ 50 kDa and p53 at ~ 53 kDa; M – indicates protein molecular weight marker.

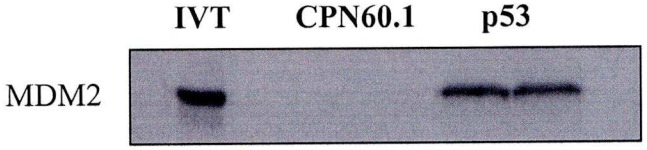


Figure 3.1.7 *In vitro* binding assay of *in vitro* translated MDM2 mixed with 6xHis-tagged p53 and CPN60.1 protein. CPN60.1 protein served as a negative control. IVT indicates 10% of the input *in vitro* translated protein. Assay was performed in duplicate as shown.

Reports on interaction between p180 and p53 have been published, showing binding of these two proteins by surface plasmon resonance analysis (13, 14). Since it has not been confirmed that p53 interacts exclusively or directly with the catalytic subunit (p180) of DNA polymerase α (13) we wanted to examine the potential direct p53 binding to p180 by performing *in vitro* binding assay.

In vitro translated full length catalytic subunit (p180) of DNA polymerase α was incubated with 6xHis-tagged proteins: CPN60.1, p53, MDM2 and in the first instance with only the amino-terminal half of MDM2. After 3 hours incubation at 30° C, samples were analyzed by SDS-PAGE and fluorography.

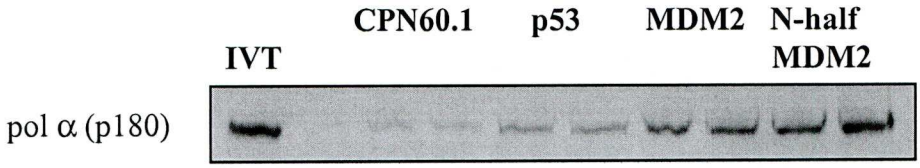


Figure 3.1.8 *In vitro* binding assay of *in vitro* translated catalytic subunit (p180) of DNA polymerase α mixed with 6xHis-tagged p53, MDM2, amino-terminal half of MDM2 and CPN60.1 protein. CPN60.1 protein served as a negative control. IVT indicates 10% of the input *in vitro* translated protein. Assay was performed in duplicate as shown.

Fluorography analysis, presented in Figure 3.1.8, revealed that the catalytic subunit of DNA polymerase α (p180) binds directly to p53, as well as to MDM2 and the amino-half of MDM2. However, comparing signals for p53 and MDM2 or p53 and the amino-terminal half of MDM2 and taking into account the amount of p53 protein present on the Ni-NTA beads (Figure 3.1.6B), it seems that p53 does not bind

to p180 as well as it does to MDM2 or the amino-terminal half of MDM2 (Figure 3.1.8).

3.1.3 *In vitro* binding assay – p180 binds preferentially to full length and amino-terminal half of MDM2

In order to test direct interactions between p180 and MDM2 proteins and identify regions involved in these interactions an *in vitro* binding assay was performed. The binding partners for the *in vitro* binding assay are presented in Table 3.1.1.

6xHis – tagged proteins	***In vitro* translated pol α (p180)**
Mouse MDM2 full length	pol α full length
	pol α N-terminal half
	pol α C-terminal half
Human MDM2 amino-terminal half	pol α full length
	pol α N-terminal half
	pol α C-terminal half
Human MDM2 carboxy-terminal half	pol α full length
	pol α N-terminal half
	pol α C-terminal half
CPN60.1	pol α full length
	pol α N-terminal half
	pol α C-terminal half

Table 3.1.1 *In vitro* binding assay pairings tested.

For these experiments p180 proteins were generated using TNT® T7 Coupled Reticulocyte Lysate System (Promega) with [^{35}S] methionine to radiolabel the proteins (as described in Materials and methods, section 2.8.1.2), while the purification of 6xHis-tagged MDM2 proteins was performed based on pilot experiments (3.1.1.1 and 3.1.1.2). Controls for the assay were included, as follows:

in vitro translated, radiolabeled fragment of DNA polymerase ε (clone 65, described in section 2.1) served as positive control for binding to MDM2 (58), while as negative control for binding to 6xHis-tagged proteins a CPN60.1 fusion protein was used (described in Materials and methods, section 2.1). Prior to use in the *in vitro* binding assay, the presence of 6xHis-tagged proteins on the beads was confirmed by Brilliant Blue G staining and western blot analysis, an example is shown in Figures 3.1.4, 3.1.5, respectively.

Based on Brilliant Blue G staining of 6xHis-tagged proteins bound to Ni-NTA beads, the carboxy-terminal half of MDM2 protein was found to be present at a higher level than the rest of the 6 x His-tagged proteins used for the assay, as shown in Figure 3.1.4. In order to have comparable amounts of input proteins in the assay, purified 6xHis-tagged carboxy-terminal half of MDM2 protein bound to the Ni-NTA beads was diluted using "empty" Ni-NTA beads suspended in Buffer B, pH 8.0 (1:1 ratio). The effect of this was verified by SDS-PAGE followed by Brilliant Blue G staining, and as shown in Figure 3.1.9 more comparable amounts of proteins were thus available for the assay. It is noteworthy that for the *in vitro* binding assay, two variants in terms of amount of 6xHis-tagged carboxy-terminal half of MDM2 input were examined (as presented in Figures 3.1.4 and 3.1.9), and the results obtained for both variants were the same (an example is presented in Figure 3.1.10).

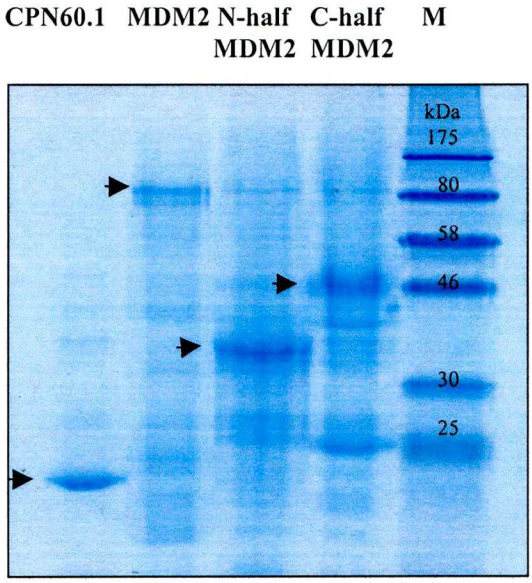


Figure 3.1.9 Brilliant Blue G staining of 6xHis-tagged proteins bound to the Ni-NTA. Proteins were separated on 15% polyacrylamide gel. Main protein bands are indicated by arrows: CPN60.1 at ~ 27 kDa, MDM2 at ~ 90kDa, amino (N)-terminal half MDM2 at ~ 42 kDa and carboxy (C)-terminal half MDM2 at ~ 50 kDa; M – indicates protein molecular weight marker. Amount of carboxy (C)-terminal MDM2 protein has been adjusted to be at a comparable level to other 6xHis-tagged proteins as shown.

An *in vitro* binding assay was performed with protein partners as presented in Table 3.1.1 (including controls) in duplicate, according to the standard protocol described in Materials and methods, section 2.8.2. Samples were analyzed by SDS-PAGE followed by fluorography and exposure of the X-ray film. Signal strength obtained on the X-ray film is an indication of the amount of direct interaction between proteins in the assay.

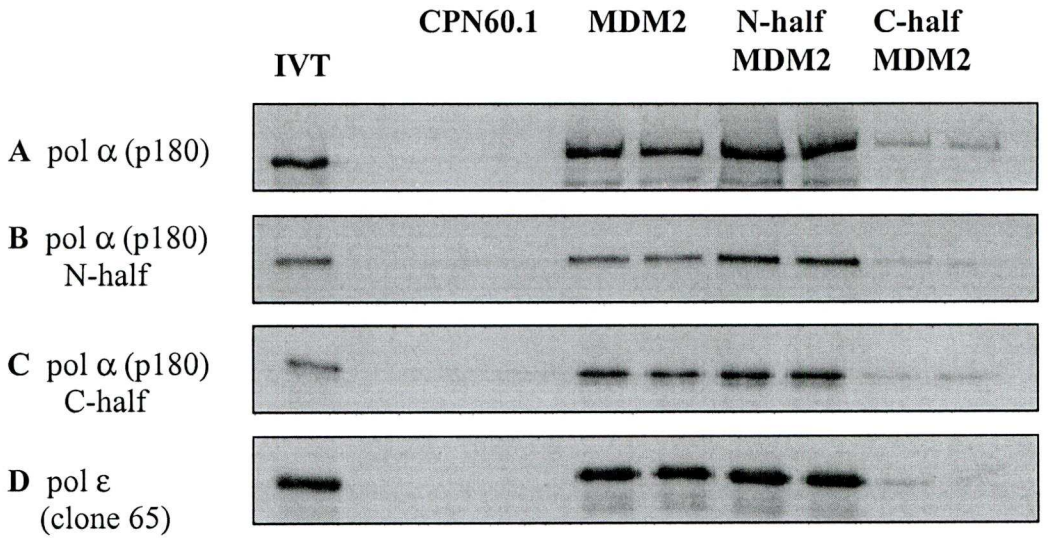


Figure 3.1.10 I*n vitro* binding assay between p180 and MDM2. I*n vitro* translated, radiolabeled: **A)** full length DNA polymerase α catalytic subunit (p180), **B)** amino (N)-terminal half of DNA polymerase α catalytic subunit (p180), **C)** carboxy (C)-terminal half of DNA polymerase α catalytic subunit (p180), **D)** carboxy (C)-terminal half of DNA polymerase ε as a positive control (clone 65), mixed with 6xHis-tagged full length mouse MDM2, amino (N)-terminal half of human MDM2, carboxy (C)-terminal half of human MDM2 and CPN60.1 protein as a negative control. IVT indicates 10% of *in vitro* translated protein input. Assays were performed in duplicate as shown.

Comparing signals obtained from the fluorographs of *in vitro* binding assays, taking into account the amount of input proteins bound to the beads for each sample, in particular the high amount of 6xHis-tagged carboxy-terminal half of MDM2 (Figure 3.1.4), it appears that the full length (FL) DNA polymerase α catalytic subunit (p180), and its amino (N)-terminal and carboxy (C)-terminal halves bind preferentially to the full length mouse MDM2 and amino (N)-terminal half of human MDM2. Despite the fact that the human carboxy (C)-terminal half of MDM2 seems to be present at the highest levels on the beads, it does not bind DNA polymerase α

catalytic subunit (p180) as well as mouse full length MDM2 or human amino (N)-terminal half of MDM2, as Figure 3.1.10 shows. Moreover, 6xHis-tagged CPN60.1, which served as a negative control does not seem to bind to p180 or its halves, while the positive control – a carboxy-terminal fragment of DNA polymerase ε (clone 65) previously shown to bind to MDM2, as expected, binds to 6xHis-tagged MDM2 and to the amino-terminal half of MDM2 (58) (Figure 3.1.10).

The results show that full length p180 as well as its amino- and carboxy-terminal halves binds directly to full length MDM2 and its amino-terminal half. To further verify these findings another *in vitro* binding assay was designed.

3.1.4 *In vitro* binding assay with Δ166 MDM2

Results obtained from *in vitro* binding assays (section 3.1.3) indicate that MDM2 interacts directly with p180, and moreover the amino-terminal half of MDM2 seems to be selectively engaged in the interaction with p180. In order to test whether the amino-terminal region of MDM2 is indispensable for direct binding with catalytic subunit of DNA polymerase α (p180) an *in vitro* binding assay was performed with a mutant of MDM2: Δ166 MDM2 lacking the amino-terminal 166 amino acids.

An *in vitro* binding assay was performed with proteins as presented in Table 3.1.2. In addition to Δ166 MDM2, full length and the amino-terminal half of MDM2 were used, as well as CPN60.1 as a negative control.

6xHis – tagged proteins	***In vitro* translated pol α (p180)**
Mouse MDM2 full length	pol α full length
	pol α N-terminal half
	pol α C-terminal half
Human MDM2 amino-terminal half	pol α full length
	pol α N-terminal half
	pol α C-terminal half
Mouse MDM2 Δ166	pol α full length
	pol α N-terminal half
	pol α C-terminal half
CPN60.1	pol α full length
	pol α N-terminal half
	pol α C-terminal half

Table 3.1.2 *In vitro* binding assay pairings tested.

Prior to the use of 6xHis-tagged proteins for the *in vitro* binding assay, their presence on the Ni-NTA beads was confirmed by SDS-PAGE analysis followed by Brilliant Blue G staining, an example is shown in Figure 3.1.11 A. It is noteworthy that based on the staining, Δ166MDM2 was present at a higher level than the other 6xHis-tagged proteins used for the assay. Therefore, an attempt to normalize the level of 6xHis-tagged proteins put into the assay was made, by diluting the Δ166MDM2 bound to Ni-NTA beads with "empty" Ni-NTA. The effect of this was verified by SDS-PAGE followed by Brilliant Blue G staining, and as shown in Figure 3.1.11 B the amounts of proteins were found to be at comparable levels. For the *in vitro* binding assay, two variants in terms of amount of Δ166MDM2 input were examined (as presented in Figures 3.1.11A and B), and the results obtained for both variants were the same (an example is presented in Figure 3.1.12).

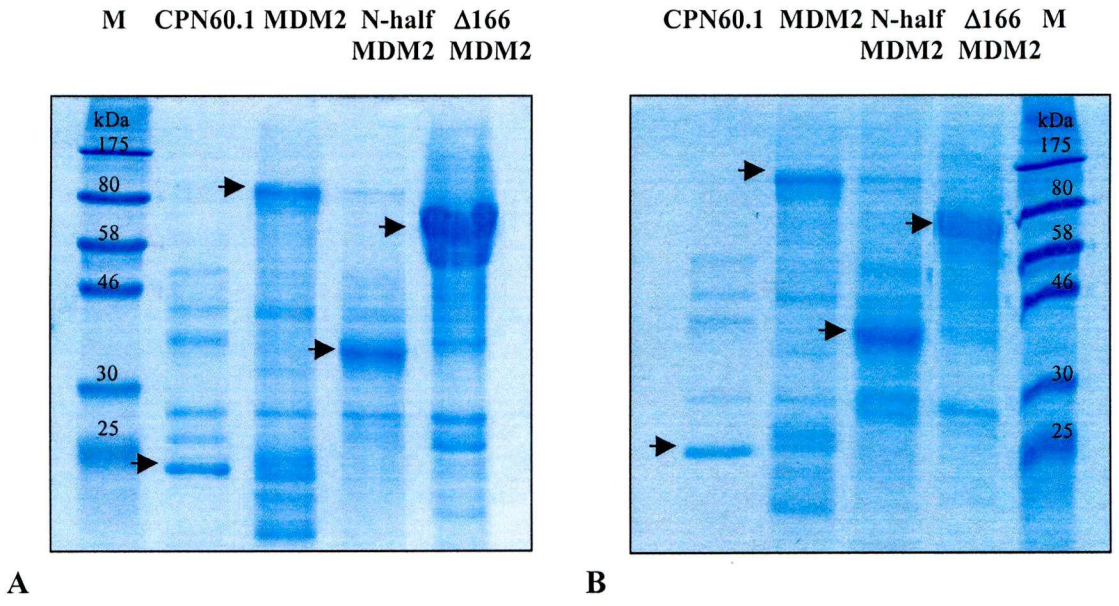


Figure 3.1.11 Brilliant Blue G staining of 6xHis-tagged CPN60.1, MDM2, amino-terminal half of MDM2 and Δ166MDM2. Brilliant Blue G staining of 6xHis-tagged proteins bound to the Ni-NTA beads after purification procedure, separated on 15% polyacrylamide gel. Main protein bands are indicated by arrows: CPN60.1 at ~ 27 kDa, MDM2 at ~ 90kDa, amino (N)-terminal half MDM2 at ~ 42 kDa and Δ166MDM2 at ~ 75 kDa; M – indicates protein molecular weight marker; **A**) represents amounts of 6xHis-tagged proteins after purification, **B**) represents adjusted amount of carboxy (C)-terminal MDM2 protein to be on comparable level to other 6xHis-tagged proteins.

An *in vitro* binding assay was performed with protein partners as presented in Table 3.1.2 (including controls) in duplicate, according to the standard protocol described in Materials and methods, section 2.8.2. Samples were analyzed by SDS-PAGE followed by fluorography and exposure of the X-ray film.

Based on results from fluorography analysis, presented in Figure 3.1.12, it appears that binding of p180 and its amino- and carboxy-terminal halves to

Δ166MDM2 is much weaker when comparing this to binding to the full length and the amino-terminal half of MDM2. This conclusion is also supported by the fact that the amount of Δ166MDM2 used in the assay was greater than the other 6xHis-tagged proteins used (Figure 3.1.11A). Preferential binding of p180 to MDM2 and its amino-terminal half confirmed previous observations (Figure 3.1.10) as did lack of binding to 6xHis-tagged CPN60.1 of p180 and its halves. Moreover, as expected the positive control for the binding assay, a fragment of DNA polymerase ε (clone 65) binds to both 6xHis-tagged MDM2 and the amino-terminal half of MDM2 (58) (Figure 3.1.12).

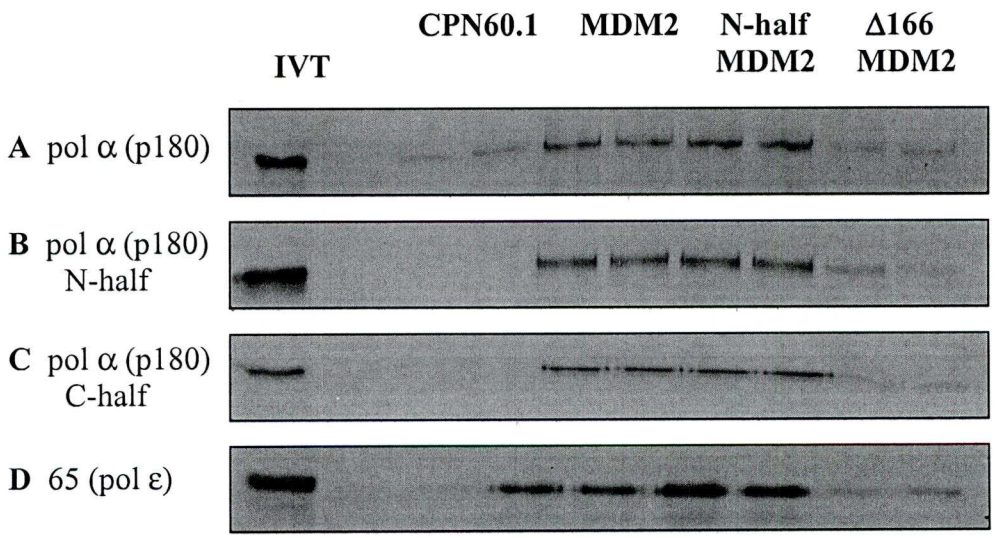


Figure 3.1.12 I*n vitro* binding assay between p180 and Δ166MDM2. *In vitro* translated, radiolabeled: **A)** full length DNA polymerase α catalytic subunit (p180), **B)** amino (N)-terminal half of DNA polymerase α catalytic subunit (p180), **C)** carboxy (C)-terminal half of DNA polymerase α catalytic subunit (p180), **D)** carboxy (C)-terminal half of DNA polymerase ε as a positive control (clone 65), mixed with 6xHis-tagged full length mouse MDM2, amino (N)-terminal half of human MDM2, mouse Δ166MDM2 and CPN60.1 protein as a negative control. IVT indicates 10% of the input *in vitro* translated protein. Assays were performed in duplicate as shown.

In summary, it can be concluded that since p180 does not seem to bind to Δ166MDM2 *in vitro*, therefore, the region of the amino-terminal 166 amino acids of MDM2 might be indispensable for direct interaction between MDM2 and p180. Thus the region of MDM2 to which catalytic subunit of DNA polymerase α binds appears to be contained between amino acids 1 and 166.

3.2 DNA polymerase α forms a complex with MDM2 in the cells

As mentioned at the beginning of the Results chapter prior to the studies described in this thesis, several experiments on MDM2 and DNA polymerase α interaction were carried out by former members of our research group – Dr. Maria Maguire and Dr. Paul Nield. One of these experiments was an immunoprecipitation assay performed on untransfected HEK293 cells showing that endogenous proteins DNA polymerase α and MDM2 forms a complex in these cells (Figure 3.2.1).

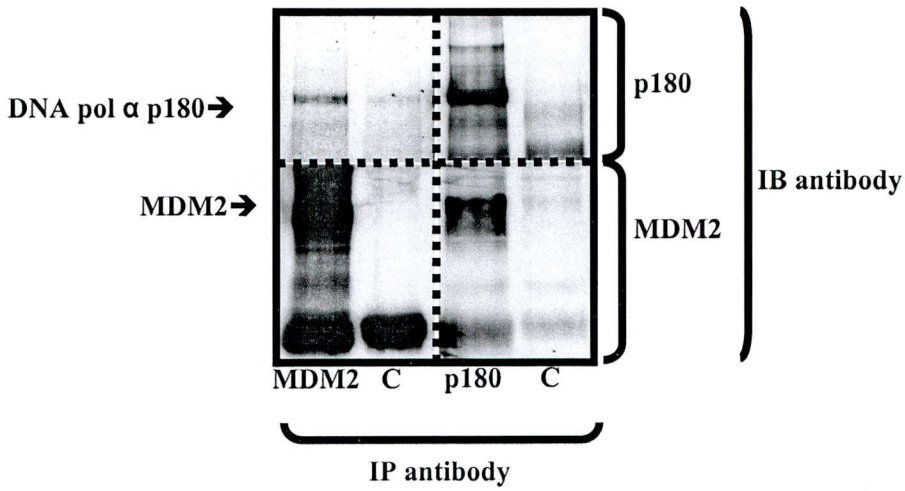


Figure 3.2.1 Co-immunoprecipitation of endogenous MDM2 with p180 from HEK293 cells. MDM2 co-precipitates endogenous DNA polymerase α and vice versa (lysates from HEK293 cells). Note that for immunoprecipitation (IP), N-19 polyclonal antibody was used for DNA polymerase α catalytic subunit, and SMP14 monoclonal antibody was used for MDM2. For immunoblot (IB) detection Ab-1 (clone IF2) monoclonal antibody was used for MDM2 and N-19 was used for DNA polymerase α catalytic subunit; C – isotype control (experiment was performed by Dr. Maria Maguire).

3.2.1 Co-immunoprecipitation experiments with MDM2 deletion mutants

Results from experiment performed by Dr. Maguire indicate that endogenous MDM2 forms a complex with DNA polymerase α in cells, as presented in Figure 3.2.1. In addition, experiments carried out *in vitro* (sections 3.1.3 and 3.1.4) revealed that amino-terminal fragment of MDM2 binds directly to p180 and region of the amino-terminal 166 amino acids of MDM2 appears to be required for that interaction with p180 (section 3.1.4). In order to determine whether such an interaction could be detected in cells in culture a series of co-immunoprecipitation (Co-IP) experiments were performed. Co-immunoprecipitation is a routinely used laboratory technique, that enables one to detect protein-protein interactions based on purification of protein complexes using a specific antibody against one of the suspected partner proteins. A weakness of this approach, however is that it cannot distinguish between direct or indirect interactions. Since we have already confirmed direct interaction between p180 and MDM2 by *in vitro* binding assay (sections 3.1.3 and 3.1.4), we were not primarily concerned in these experiments with the question of whether interactions were direct, rather we wanted to examine the regions of MDM2 required for interaction with p180 in live cells. To facilitate these studies we used N-terminal HA–tagged MDM2 carboxy-terminal deletion mutants and a schematic representation of these is shown in Figure 3.2.2.

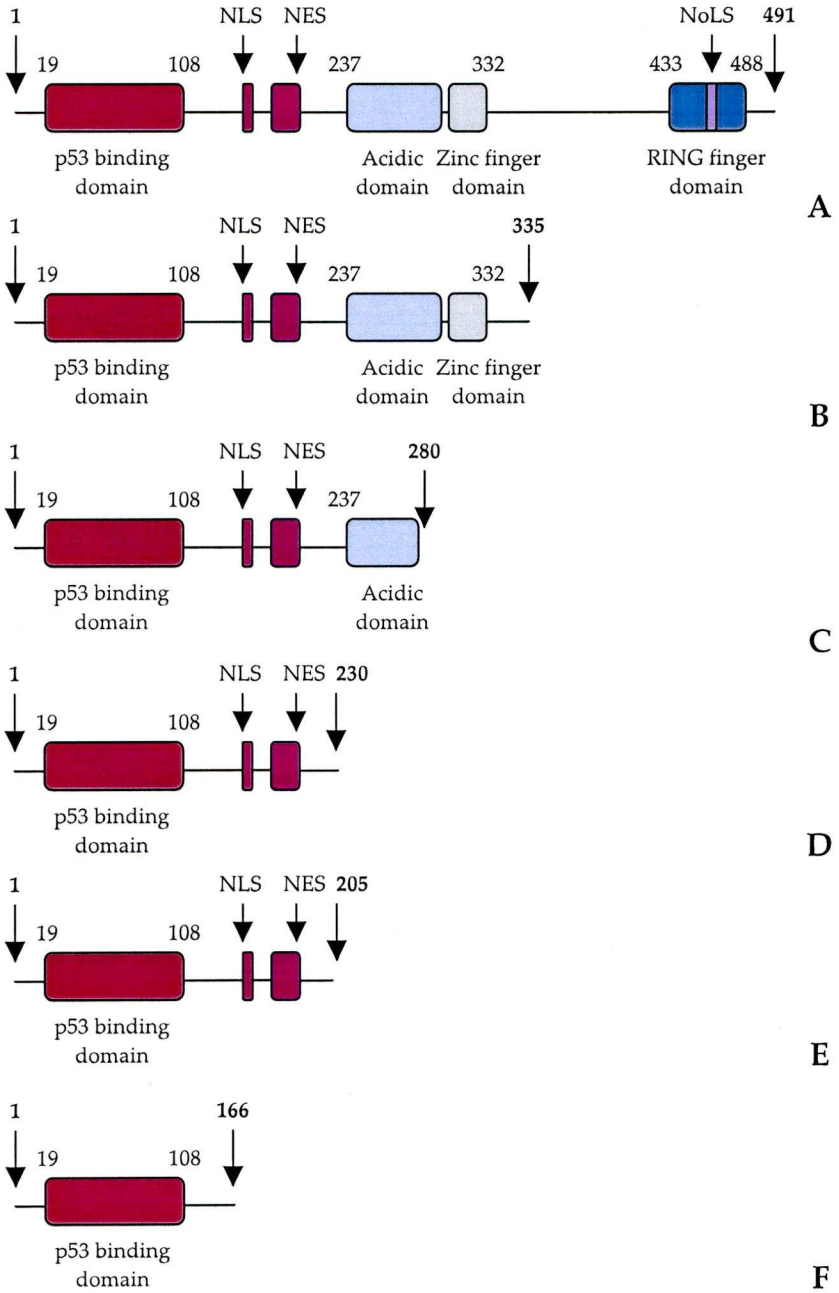


Figure 3.2.2 Schematic representation of N-terminal HA–tagged carboxy–terminal deletions MDM2 mutants used for co-immunoprecipitation experiments. Location of different domains and motifs indicated by coloured boxes; NLS – nuclear localization signal, NES – nuclear export signal, NoLS – nucleolar localization signal. Constructs were made by a former member of our research group - Dr. Tim Devling.

3.2.1.1 An examination of the expression levels of HA-MDM2 carboxy-terminal deletion mutants

Prior to performing a Co-IP experiment with HA-MDM2 mutants (Figure 3.2.2), an examination of their expression levels was carried out. It is desirable that the constructs used for transient tranfection in Co-IP experiments express proteins at comparable levels in order to be able to properly analyze obtained results, since Co-IP results obtained for each construct are then compared against each other. Therefore, H1299 cells were transfected, using GeneJuice™ (at a 3:1 v:w ratio to DNA), with 10 μg of plasmid DNA from each of the HA-MDM2 mutant constructs. 24 hours after transfection cells were harvested, lysed in SLIP buffer and protein concentration in the lysates was measured using the Bradford method (as described in section 2.6). Normalized amounts of proteins from each transfection were separated by 12% SDS-PAGE followed by western blot analysis. Anti-MDM2 antibody (Ab1, clone IF2) was then used to detect MDM2 carboxy–terminal deletion mutants.

Based on the results obtained from western blot analysis, all the constructs express MDM2 proteins (Figure 3.2.3B). Comparing signals amongst MDM2 carboxy–terminal deletion mutants it was apparent that the mutant protein consisting of the amino terminal 335 amino acids was expressed at lower levels than the other mutants. Examination of the Ponceau S stained membrane (Figure 3.2.3A), which served as a loading control, demonstrated no obvious difference in loading of the samples on the gel. The transfection efficiency based on western blot analysis (Figure 3.2.3C), was also comparable for each construct. Thus it appears that mutant 335 possesses an intrinsically reduced ability to produce protein.

Nevertheless, since all the constructs detectably express MDM2 mutant proteins the next stage of the experiment could be performed.

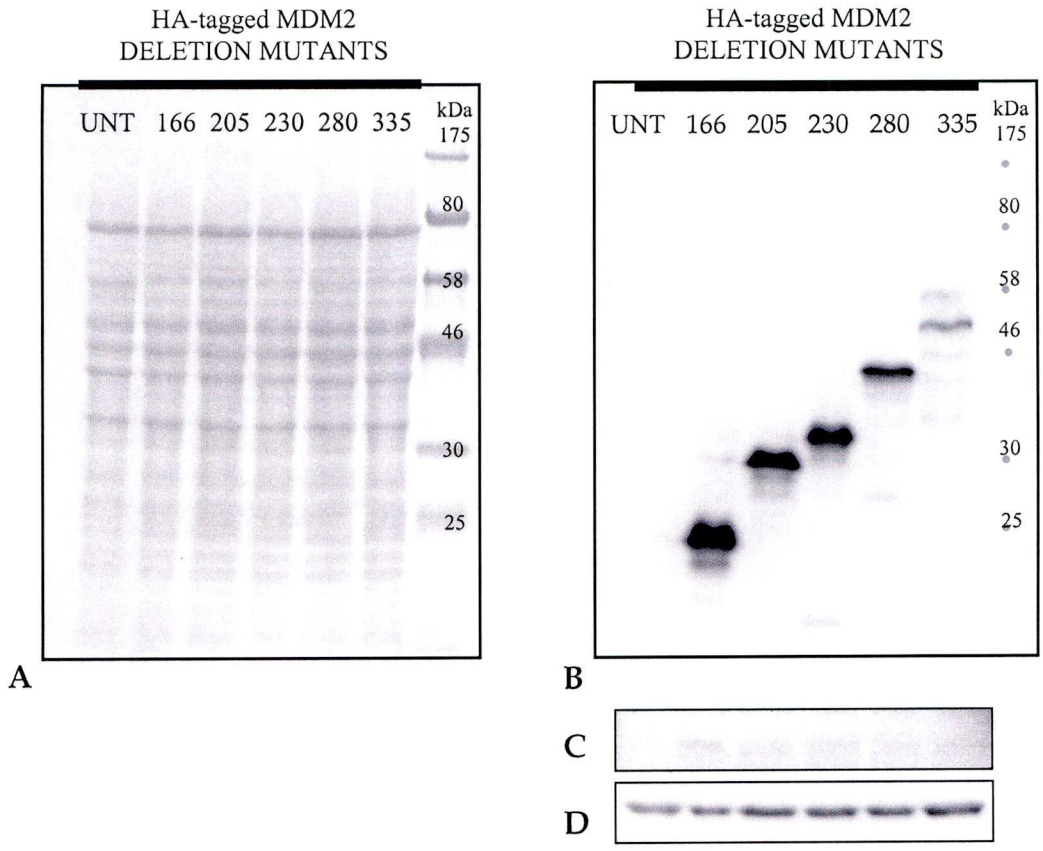


Figure 3.2.3 Test of expression of HA – tagged MDM2 carboxy-terminal deletion mutants. Proteins were separated on 12% polyacrylamide gel: **A)** Ponceau –S staining, **B)** western blot with anti-MDM2 antibody (Ab1, clone IF2), **C)** western blot with anti-β-galactosidase antibody (Ab1, clone 200-193), **D)** western blot with anti-actin antibody (C-2) (loading control for β-galactosidase).

3.2.1.2 Determination of conditions for immunoprecipitation experiments with HA-MDM2 carboxy-terminal deletion mutants

After testing expression levels of HA-MDM2 carboxy-terminal deletion mutants another experiment was performed in order to establish conditions for the immunopreciptation procedure. Two different antibodies were examined – anti-HA tag (16B12) and anti-DNA polymerase α catalytic subunit p180 (N-19) in order to be used in subsequent immunoprecipitation experiments.

Therefore, H1299 cells were transfected with expression vectors for p180 – the catalytic subunit of DNA polymerase α, β-galactosidase and either the 166 or 335 amino acids fragment of HA-tagged MDM2 mutant. Immunoprecipitation was carried out as described in Materials and methods (section 2.10) and two antibodies were tested: an antibody recognizing HA tag (16B12) was used for one set of lysates, while an antibody recognizing the DNA polymerase α catalytic subunit p180 (N-19) was used on the other set. Appropriate antibodies were also included as isotype/antibody controls: anti-CD20 (Leu16) and anti–DHFR (E-18), respectively.

Samples from immunoprecipitation experiments were analysed by SDS-PAGE followed by western blotting with anti-p180 (N-19) and anti-MDM2 (Ab1, clone IF2) antibodies (Figure 3.2.4A-E). Based on the results from western blot analysis, it is clear that immunoprecipitation with an anti-p180 antibody resulted in co-precipitation of p180 with both 166 and 335 HA-MDM2 mutants, as presented in Figure 3.2.3C. However, when anti-HA tag (16B12) antibody was used for precipitation, only HA-tagged MDM2 mutants were immunoprecipitated and p180 could not be detected (Figure 3.2.4B).

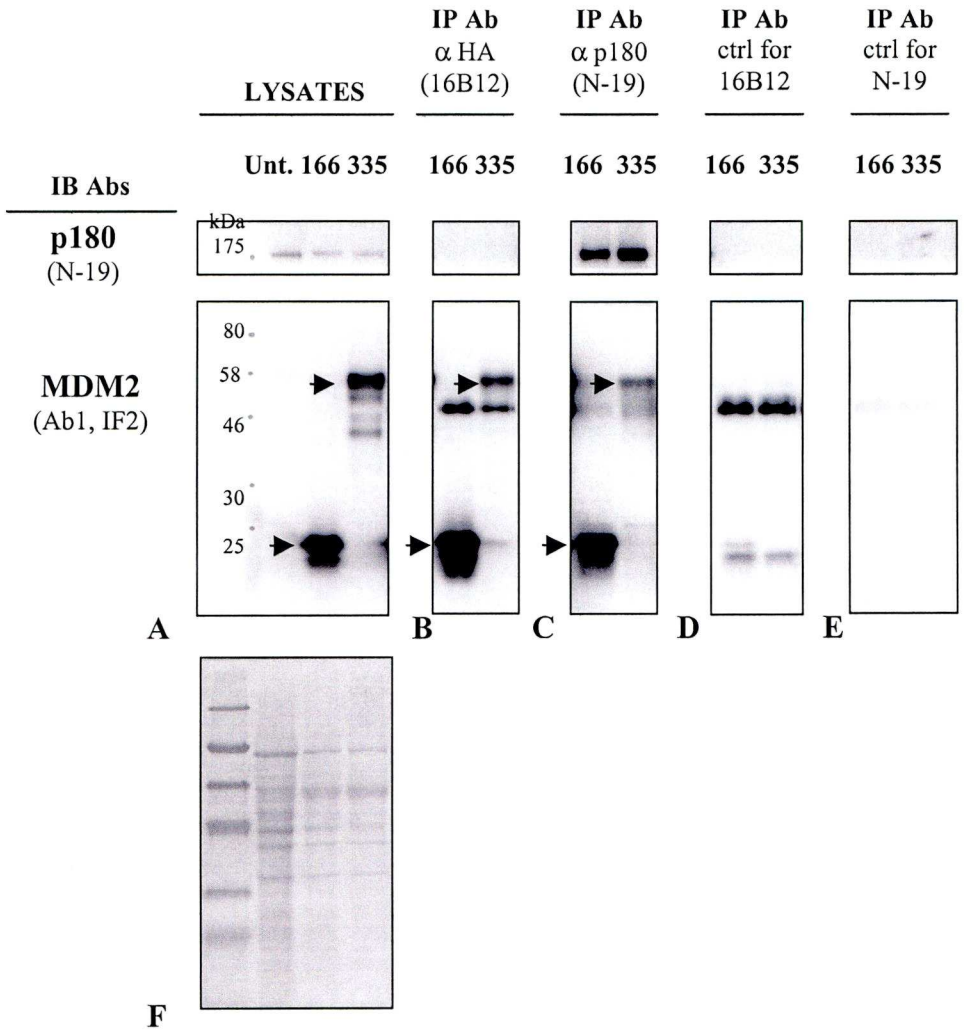


Figure 3.2.4 Co-immunoprecipitation experiment of p180 with 166 and 335 HA-tagged MDM2. H1299 cells were transfected with p180 (30 μg), β-galactosidase (6 μg) and either 166 HA-MDM2 or 335 HA-MDM2 (30 μg) (**A**). Two 15 cm dishes of H1299 cells per each condition were transfected with GeneJuice™ reagent at a 3:1 ratio. For each immunoprecipitation 3 mg of protein lysate were used. For immunoprecipitation the antibodies used were: **B**) anti-HA (16B12) antibody (2μg); **C**) anti-p180 (N-19) antibody (2μg); **D**) anti-CD20 (Leu16) (2μg) - antibody control for anti-HA (16B12) antibody; **E**) anti-DHFR (E18) (2μg) - antibody control for anti-p180 (N-19) antibody; **F**) Ponceau-S stained membrane presented as a loading control. Mutant MDM2 proteins are indicated by arrows: 166 ~ at 22 kDa, 335 ~ at 54 kDa (**A**, **B**, **C**).

Based on results presented in Figure 3.2.4 we concluded that the anti-p180 antibody could be used for further immunoprecipitation experiments with p180 and HA-tagged MDM2 mutant proteins, while the anti-HA tag (16B12) antibody was not suitable for this purpose. Furthermore, as shown in Figure 3.2.4B, 166 and 335 HA-tagged MDM2 were co-immunoprecipitated with p180 protein, thus it appears that these proteins form complexes with DNA polymerase α in cells.

3.2.1.3 Co-immunoprecipitation of p180 with Δ 1-49, 166, 230, 280 MDM2

The experiment described in section 3.2.1.2 demonstrated that the anti-p180 antibody (N-19) was suitable for Co-IP experiments with DNA polymerase α catalytic subunit (p180) and MDM2 mutants, thus we decided to use that antibody to further examine p180-MDM2 interactions in cells in culture. In addition to the HA-tagged MDM2 mutants (presented in Figure 3.2.2) another MDM2 deletion mutant (Δ1-49 MDM2) lacking the first 49 amino acids of MDM2 (see schematic representation in Figure 3.2.5) was available in our laboratory (described in more detail in reference no. 57) and therefore we decided to include that mutant in our immunoprecipitation experiment.

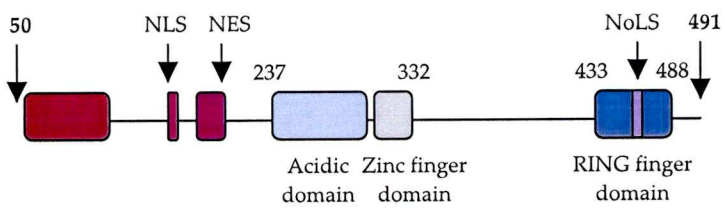


Figure 3.2.5 Schematic representation of MDM2 mutant lacking the amino-terminal 49 amino acids. Construct was made by former member of our research group - Dr. Mark Brady (57).

As with the other mutants, prior to using the Δ1-49 MDM2 construct in immunoprecipitation experiments, the expression levels of Δ1-49 MDM2 and 166 HA-MDM2 were compared in cell lysates of transiently transfected H1299 cells by SDS-PAGE and western blot. As shown in Figure 3.2.6 the expression level of both constructs was comparable, hence the Δ1-49 MDM2 construct was deemed to be suitable to use alongside 166 HA-MDM2 and other HA-MDM2 carboxy-terminal deletion mutants in subsequent immunoprecipitation assays.

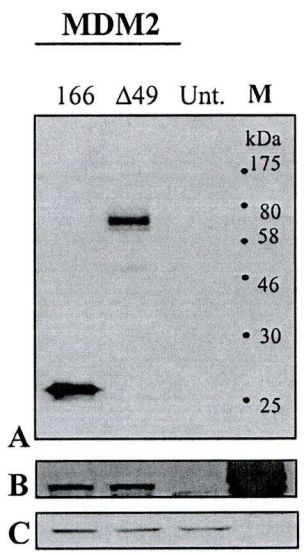


Figure 3.2.6 Test of expression of 166 and Δ49 MDM2 mutants. H1299 cells were transfected with 10 μg of the appropriate MDM2 plasmid DNA and 2 μg of β-galactosidase plasmid using GeneJuice™; **A)** western blot with anti-MDM2 antibody (Ab1, clone IF2), **B)** western blot with anti-β-galactosidase antibody (Ab1, clone 200-193) (transfection efficiency control), **C)** western blot with anti - actin antibody (C-2) (loading control); M – indicates protein molecular weight marker.

H1299 cells were transfected with expression vectors for p180, β-galactosidase and either Δ1-49, 166, 230 or 280 MDM2 per each condition. Cells were then processed as described in Materials and methods (section 2.10) and

samples analysed by SDS-PAGE followed by western blot analysis with anti-p180 (N-19) and anti-MDM2 (Ab1, clone IF2) antibodies (Figure 3.2.7).

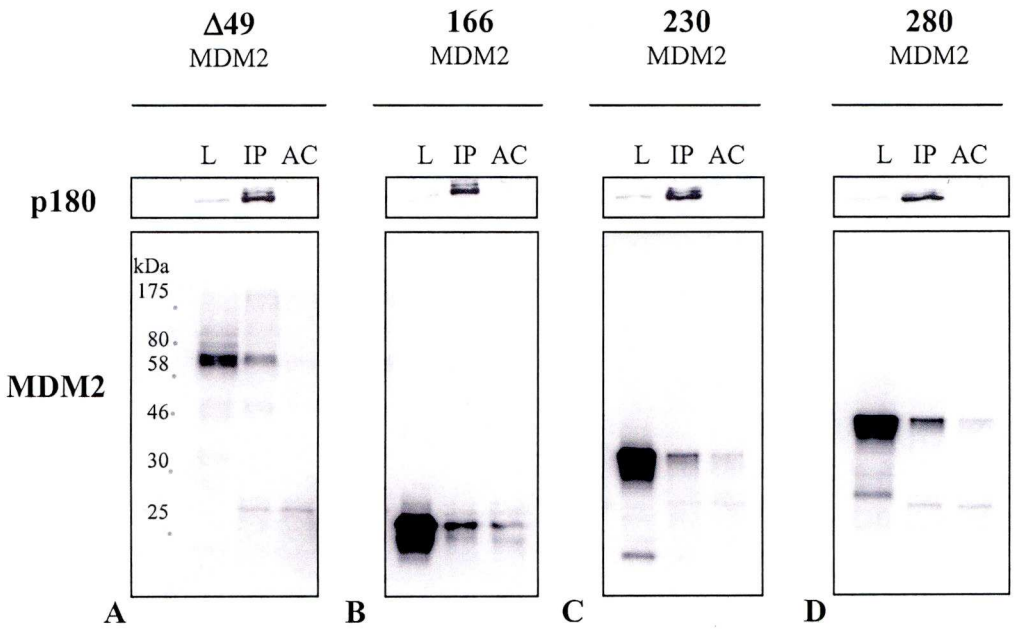


Figure 3.2.7 Co-immunoprecipitation of p180 with Δ49, 166, 230 and 280 MDM2. H1299 cells were transfected with p180 (30 μg) and either Δ49MDM2 (30 μg) (lane L, panel **A**), 166HA-MDM2 (30 μg) (lane L, panel **B**), 230HA-MDM2 (30 μg) (lane L, panel **C**) or 280HA-MDM2 (30 μg) (lane L, panel **D**). Experiment was carried out using 3mg of protein, immunoprecipitation was performed with anti-p180 (N-19) antibody (lanes marked: IP) and anti-DHFR (E18) as an antibody control (lanes marked: AC). For western blot analysis p180 (N-19) and MDM2 (Ab1, clone IF2) antibodies were used.

Results obtained after immunoblot analysis confirmed that p180 can be co-immunoprecipitated with 166 HA-MDM2 mutant (Figures 3.2.4C and 3.2.7B). In addition, Δ1-49, 230 and 280 MDM2 mutants were also co-immunoprecipitated with p180 (Figure 3.2.7 A,C,D).

Based on these findings, it appears that the MDM2 region involved in forming a complex in the cell with p180 lies between amino acids 50 and 166 of MDM2. In order to further test this conclusion, an MDM2 mutant lacking the amino-terminal 166 amino acids was generated that could be used to drive expression in mammalian cells, and subsequently examined for the ability of the mutant MDM2 to co-immunoprecipitate with p180.

3.2.1.4 The amino terminal 166 amino acids fragment of MDM2 is dispensable for forming a complex with p180 in cells in culture

Immunoprecipitation experiments performed thus far revealed that p180 forms complexes in cells with MDM2 deletion mutants, as presented in Figures 3.2.4 and 3.2.7. Based on the immunoprecipitation data, the region between amino acids 50 and 166 of MDM2 seems to mediate the interaction with p180. To further examine this possibility, an MDM2 mutant lacking the amino-terminal 166 amino acids (Δ166MDM2) was created as described in Materials and methods (section 2.1); schematic representation of Δ166MDM2 is presented in Figure 3.2.8.

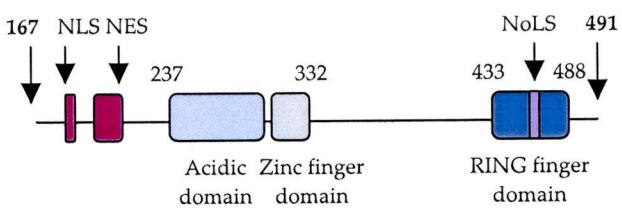


Figure 3.2.8 Schematic representation of MDM2 mutant lacking of the amino-terminal 166 amino acids. Location of different domains and motifs indicated by coloured boxes; NLS – nuclear localization signal, NES – nuclear export signal, NoLS – nucleolar localization signal.

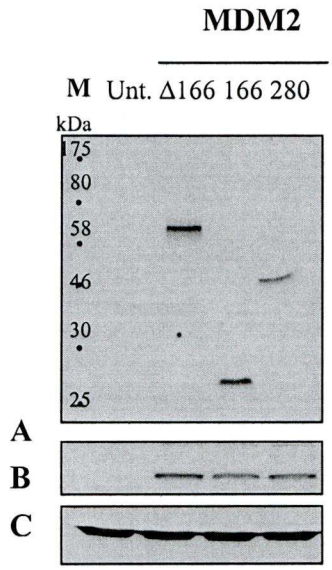


Figure 3.2.9 Test of expression of Δ166, 166 and 280 MDM2 mutants. H1299 cells were transfected with 10 μg of appropriate MDM2 plasmid DNA and 2 μg of β-galactosidase using GeneJuice™ and separated along untransfected H1299 cells lysate (Unt.); **A)** western blot with anti-HA antibody (16B12), **B)** western blot with anti-β-galactosidase antibody (Ab1, clone 200-193) (transfection efficiency control), **C)** western blot with anti-actin antibody (C-2) (loading control for β-galactosidase); M – indicates protein molecular weight marker.

Before using the Δ166MDM2 construct in immunoprecipitation experiments, the expression levels of Δ166MDM2 and for comparison the existing 166 and 280 HA-MDM2 mutants were compared in transiently transfected H1299 cells analyzed by SDS-PAGE and western blot. As shown in Figure 3.2.9 the expression level of constructs was at comparable levels, hence the Δ166MDM2 construct was deemed suitable to use alongside the 166, 280 HA-MDM2 and other MDM2 deletion mutants in subsequent immunoprecipitation assays.

H1299 cells were transfected, using Genejuice, with expression vectors for p180, β-galactosidase and either Δ1-49, Δ166, 166, 230, 280 or 335 MDM2 mutants. Immunoprecipitation was carried out as described in Materials and methods (section

2.1), then samples were analysed by SDS-PAGE followed by western blot analysis with anti-p180 and either anti-MDM2 (Ab1, clone IF2) (Figure 3.2.10A) or anti-HA (16B12) (Figure 3.2.10B).

As shown in Figure 3.2.10A, results for already tested MDM2 mutants were in agreement with previous findings with Δ1-49, 166, 230, 280 and 335 MDM2 were all found to immunoprecipitate with p180 (c.f. Figures 3.2.4C, 3.2.7). However, the Δ166 MDM2 was also found to immunoprecipitate with p180 (Figure 3.2.10B, IP panel). This observation suggests that whilst the region of amino-terminal 166 amino acids of MDM2 is required for direct interaction with p180 *in vitro*, it is dispensable for complex formation with p180 in the cell. Co-immunoprecipitation assays cannot distinguish between direct and indirect interaction between proteins and thus these results may be due to MDM2 and p180 interacting with other proteins in a complex in the cell.

Results from *in vitro* binding assay (sections 3.1.3, 3.1.4) indicated that p180 and MDM2 interact directly, with the amino-terminal 166 amino acids of MDM2 being required for *in vitro* interaction. However, *in vivo* experiments have not confirmed an essential role of this amino-terminal 166 amino acids of MDM2 in mediating interaction with p180 in cells, albeit that the existence of a protein complex involving p180 and MDM2 in cells was observed. Based on the results obtained, we conclude that p180 and MDM2 interact directly and that the amino-terminal 166 amino acids of MDM2 are required for direct interaction with p180 *in vitro*. In addition, p180 and MDM2 interact in cells in culture, but incorporation of MDM2 into this complex does not depend upon the amino-terminal 166 amino acids of MDM2 and may well depend upon other protein/s.

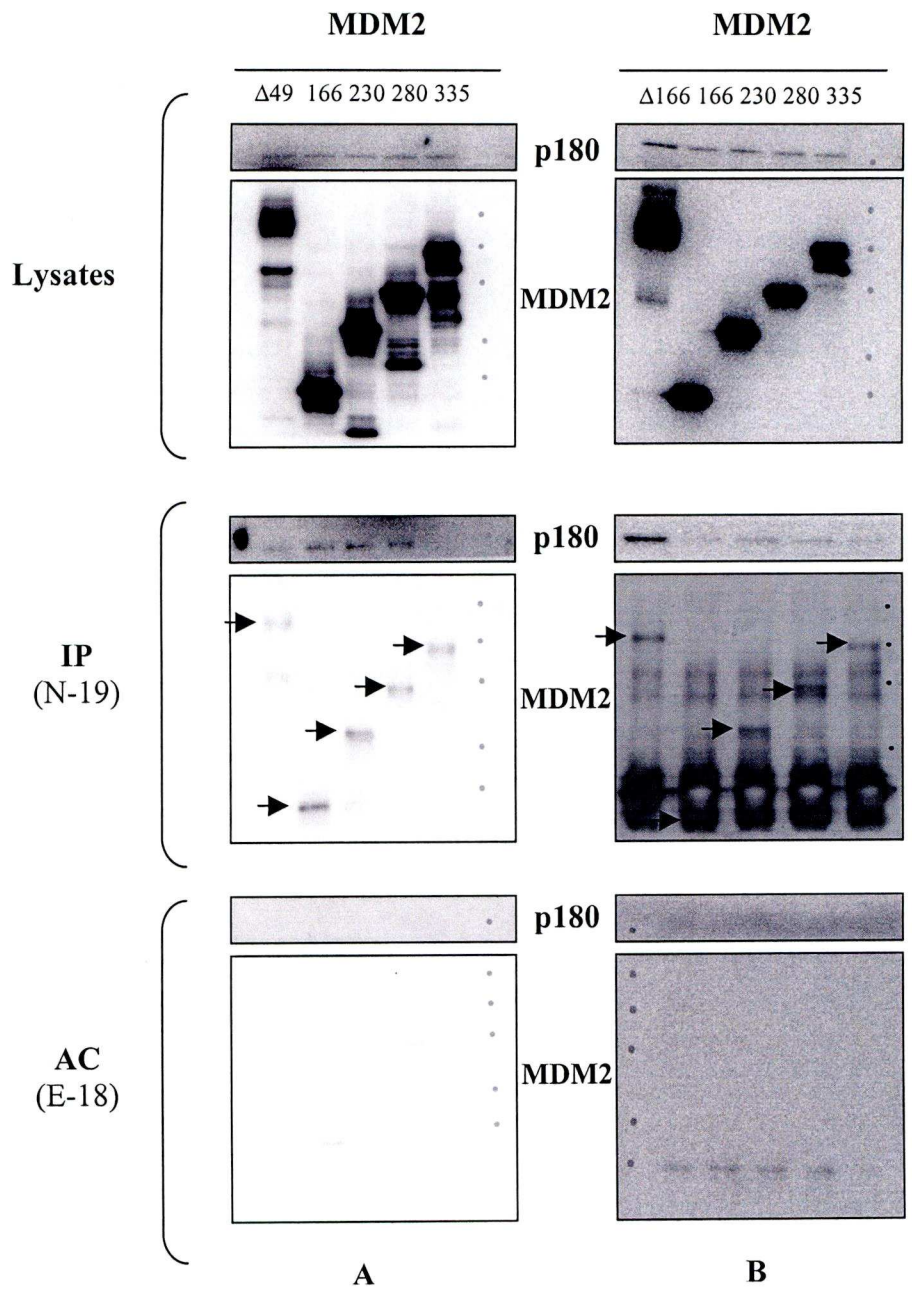


Figure 3.2.10 Co-immunoprecipitation of p180 with MDM2 mutants. H1299 cells were transfected with p180 (30 μg) and either Δ49, Δ166, 166, 230, 280, 335 MDM2 (30 μg) using GeneJuice™ reagent, as presented in Lysates panel. Immunoprecipitation was performed on 3mg of total protein with anti-p180 (N-19) antibody (IP panel) and anti-DHFR (E18) as an antibody control (AC panel). For western blot analysis anti-p180 (N-19) and anti-MDM2 (Ab1, clone IF2) antibodies were used in section **A**, in section **B** anti-p180 (N-19) and anti-HA (16B12). Main MDM2 mutants bands indicated by arrows (IP panel): Δ49 at ~ 77 kDa, 166 at ~ 22 kDa, 230 at ~ 30 kDa, 280 at ~ 42 kDa, 335 at ~ 55 kDa, Δ166 at ~ 65 kDa.

3.3 Examining the effect of MDM2 on DNA polymerase α activity

Results obtained from *in vitro* binding assay and immunoprecipitation experiments revealed that MDM2 interacts with the catalytic subunit of DNA polymerase α, both *in vivo* and *in vitro* (sections 3.1, 3.2). When studies on interaction between MDM2 and DNA polymerase ε were performed by the members of our research group (58, 59) they revealed that the polymerase activity of DNA polymerase ε was stimulated by MDM2 *in vitro* (59). Encouraged by these findings we decided to perform similar experiments in order to test whether MDM2 may stimulate or otherwise modify the catalytic activity of DNA polymerase α.

3.3.1 Purification of the catalytic subunit (p180) of DNA polymerase α

In order to perform DNA polymerase assays to examine DNA polymerase α activity upon addition of MDM2 we first needed to produce and purify the catalytic subunit (p180) of DNA polymerase α. The purification of 6xHis-tagged p180 was performed using Ni-NTA agarose beads under native conditions in order to retain p180 enzymatic activity.

DNA polymerase α catalytic subunit (p180) was expressed in and purified from insect cells using procedures described in Materials and methods (section 2.9). Cells lysates, after infection with pBlueBacHis2p180, were incubated with Ni-NTA beads, the lysate-Ni-NTA bead mixture was then applied to a column. In order to determine the amount of the specific protein that has not bound to the beads, the column flow-through fraction was collected. The beads on the column were extensively washed with SLIP buffer containing 20mM imidazole, to remove proteins nonspecifically

bound to Ni-NTA beads. After washing, the catalytic subunit (p180) of DNA polymerase α was gradually eluted using SLIP buffer containing increasing amounts of imidazole: 50, 100, 150 and 200 mM imidazole.

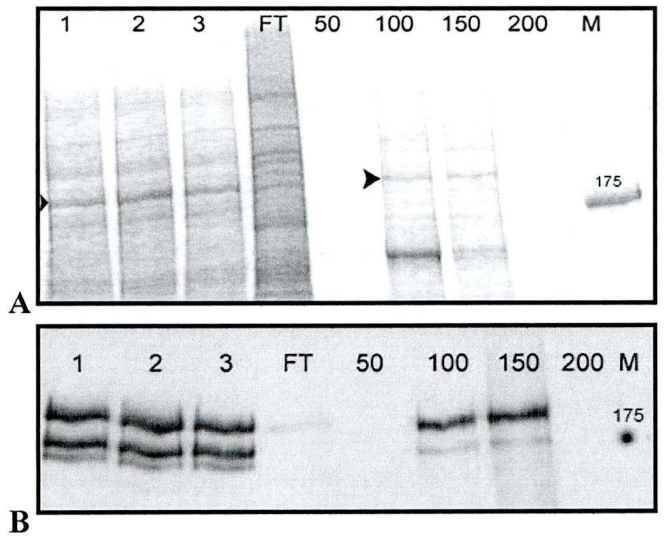


Figure 3.3.1 Brilliant Blue G staining and western blot analysis of fractions collected during p180 purification from the insect cells. Lanes marked 1–3 represent fractions of 0.03% of raw lysates from insect cells cultures; FT represents 0.03% of flow through from the column; 50 – 200 lanes represent 2% of elution/imidazole (mM) fractions. Fractions were separated on 6% polyacrylamide gel, followed by: **A)** staining with Brilliant Blue G, arrows indicate 6xHis-tagged catalytic subunit (p180) of DNA polymerase α location on the gel at ~ 180kDa, **B)** western blot analysis using anti DNA polymerase α (N – 19) antibody; M – indicates protein molecular weight marker.

Fractions obtained during p180 purification (including raw lysates of insect cells, column flow- through and elution fractions) were subjected to 6% SDS-PAGE followed by Brilliant Blue G staining and western blot analysis. As shown in Figure 3.3.1 A and B, DNA polymerase α protein is present in fractions from raw lysates,

which indicate successful infection of the insect cells with virus from pBlueBacHis2p180 transfected insect cells. In addition, the amount of p180 in the flow through fraction indicating the amount of unbound protein was relatively low, which suggested that majority of protein of interest has bound to the Ni-NTA beads. Finally, the presence of p180 in elution fractions with 100 and 150 mM imidazole, is also indicative of successful purification.

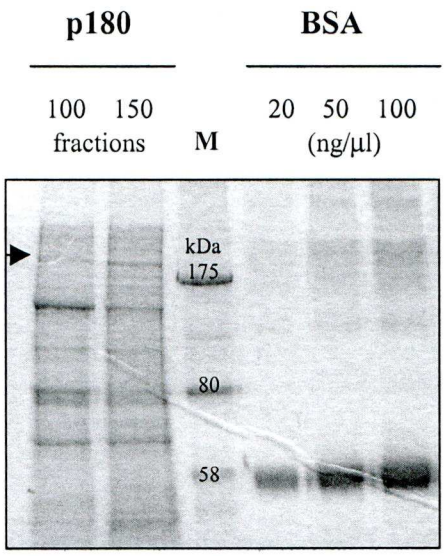


Figure 3.3.2 Brilliant Blue G staining of 100 and 150 mM imidazole fractions of p180 fractions. Fractions represent 100 and 150 mM imidazole eluents collected during DNA polymerase α purification process separated along with increasing amount of BSA; arrow indicates DNA polymerase α location on the gel at ~ 180kDa; M – indicates protein molecular weight marker.

After purification of DNA polymerase α catalytic subunit (p180), we wanted to estimate the amount of p180 protein present in 100 and 150 mM imidazole fractions, thus both samples along with increasing amount of BSA as a standard were separated on 8% polyacrylamide gel and stained with Brilliant Blue G (Figure 3.3.2). Based on the Brilliant Blue G staining, the amount of protein of interest

recovered was compared to a BSA standard and although the standard appeared to be overloaded, it was estimated that 100 mM fraction contains approximately 5 ng per µl, whereas 150 mM about 10 ng per µl. For the further experiments p180 from the 150 mM fraction were used, since the amount of protein was greater than in 100 mM fraction.

3.3.2 Examination of p180 enzymatic activity

Pilot DNA polymerase assays were performed in order to test whether the enzymatic activity of p180 was retained after purification from insect cells. The assay in general enables one to measure polymerase activity based on incorporation of radiolabeled nucleotides e.g. [^{3}H] dTTPs.

The time course experiment was performed with different amounts of catalytic subunit of DNA polymerase α (p180) from 150 mM imidazole elution fraction (Table 3.3.1) assayed as described in Materials and methods (section 2.13) and then collected at several time points as specified in Table 3.3.1.

Amount of p180 (ng)	Time points (minutes)
10	0, 15, 30, 60, 120
20	
40	

Table 3.3.1 Conditions for DNA polymerase assay – time course. Amounts of p180 used and the time points at which samples were collected are indicated.

Reaction products were TCA-precipitated onto filters, air-dried, suspended in scintillant and counted. Results are shown in Figure 3.3.3 which demonstrates that

that p180 purified from the insect cells retains enzymatic activity in this assay. The rate of product formation is almost linear during the first 15 minutes, then decreases, however the enzyme retains detectable activity up to 2 hours. The figure also demonstrates that there is a dose-dependent incorporation of [3H]dTTPs.

Based on these results, we conclude that the enzymatic activity of DNA polymerase α catalytic subunit (p180) purified from insect cells has been retained, and that by performing assays for 1 hour should provide a good compromise between a stable linear reaction rate and assay sensitivity.

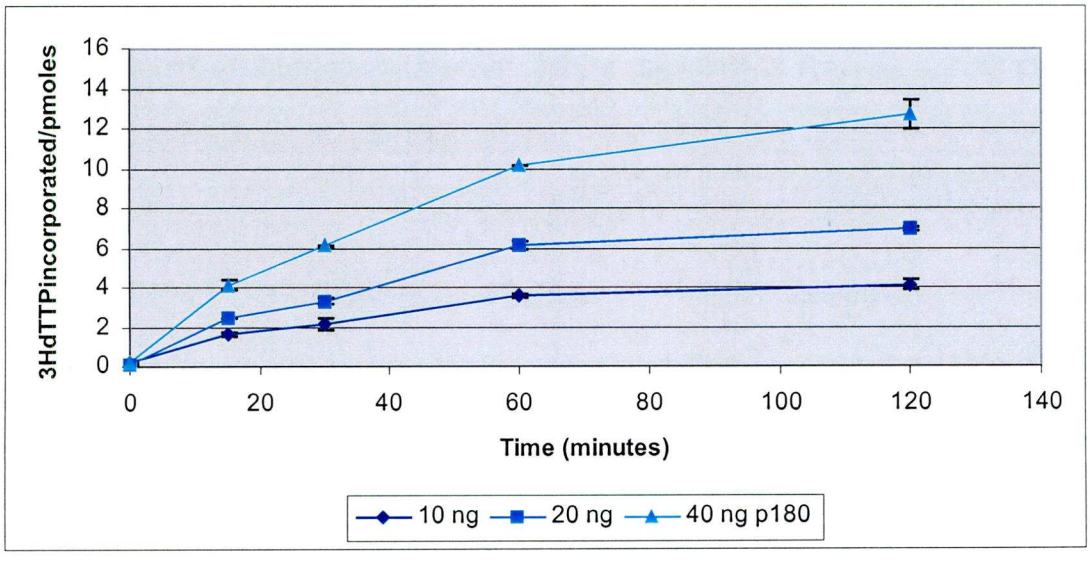


Figure 3.3.3 Enzymatic activity of purified 6xHis-tagged human DNA polymerase α catalytic subunit - p180 expressed in baculovirus infected insect cells. Three different amounts of p180 (10, 20 and 40 ng) were assayed and collected at certain time points (0, 15, 30, 60, 120 minutes). Incorporation of [3H]dTTP (picomoles) was measured by scintillation counting. Data are shown as mean ± standard error of the mean.

3.3.3 DNA polymerase assay with MDM2 purified from the insect cells

Once the enzymatic activity of purified p180 was confirmed in the pilot assay (section 3.3.2), other tests were performed in order to investigate whether MDM2 influences p180 polymerase activity.

For these experiments MDM2 from insect cells purified by a former member of our research group - Dr. Eider Larrarte was used. Aliquots of MDM2 were stored at –80°C and each contained approximately 60 ng per µl of MDM2 protein. The MDM2 was produced in insect cells after infection with a baculovirus vector and the purification procedure was conducted in the same way as described for p180 in Materials and methods (section 2.9).

Amount of p180	**Amount / volume of MDM2**	**Volume of assay buffer**
1 ng	-	9 µl
3 ng		
10 ng		
1 ng	540 ng/9 µl	-
3 ng		
10 ng		

Table 3.3.2 Conditions for DNA polymerase assay with MDM2 purified from insect cells. Different amounts of p180 were assayed with either specified amount/volume of MDM2 or assay buffer as indicated.

To examine any putative MDM2 effect on polymerase activity of p180, a DNA polymerase assay was performed, with p180 titrated in the presence of either

MDM2 or the assay buffer, as presented in Table 3.3.2. After one hour incubation at 37°C, samples were transferred to filters, washed and counted in the scintillation counter.

The results shown in Figure 3.3.4 demonstrate that amount of incorporated [3H]dTTPs is higher when p180 is assayed in the presence of MDM2 than for p180 assayed with only the buffer. For each of three different amounts of p180 used, there is a noticeable difference ranging from approximately two-fold higher values for 10 ng of p180 to almost 3.5 fold higher activity for 3 ng of p180.

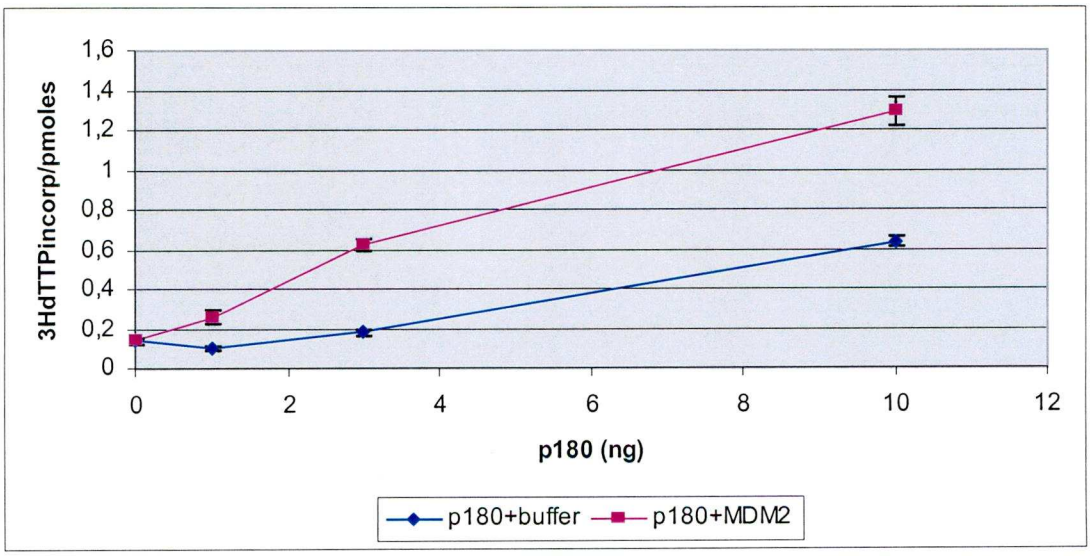


Figure 3.3.4 Enzymatic activity of purified 6xHis-tagged p180 upon addition of MDM2 purified from insect cells. DNA polymerase α catalytic subunit - p180 expressed in baculovirus infected insect cells mixed with either assay buffer (p180+buffer) or MDM2 (p180+MDM2). Three different amounts of p180 (1, 3 and 10 ng) were assayed with buffer/MDM2 and collected after 60 minutes. Incorporation of [3H]dTTP (picomoles) was measured by scintillation counting. Data are shown as mean ± standard error of the mean.

These observations, based on results presented in Figure 3.3.4, suggest that addition of MDM2 stimulates the polymerase activity of p180 *in vitro*. However, the possibility exists that MDM2 expressed and purified from the insect cells might either possess or co-purify with DNA polymerase activity.

During the protein purification process there is the possibility that contaminants that possess DNA polymerase activity or other proteins that may affect activity of assayed enzyme bind to the resin/protein and then are eluted with the protein of interest, we were concerned about the purity of MDM2 protein purified from the insect cells and by the possibility of such contaminating enzymatic activity. In order to address these questions, we prepared equivalent fractions of protein from uninfected insect cells, grown and processed in exactly the same manner as used for MDM2 protein purification procedure (Figure 3.3.5). The presence of p180 and MDM2 in uninfected cells fractions was verified by western blot with anti-DNA polymerase α and anti-MDM2 antibodies, respectively (Figure 3.3.6).

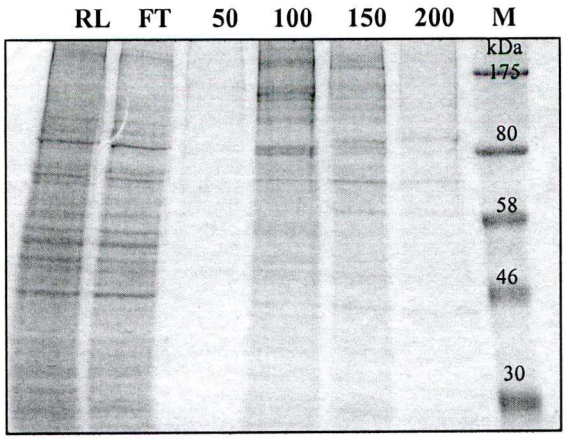


Figure 3.3.5 Brilliant Blue G staining of fractions collected during purification process of uninfected insect cells. Lanes marked: RL represents 0.03% raw lysate from insect cells culture; FT represents 0.03% of flow through from the column; 50 – 200 lanes represent 2% of elution/imidazole (mM) fractions; M – indicates protein molecular weight marker.

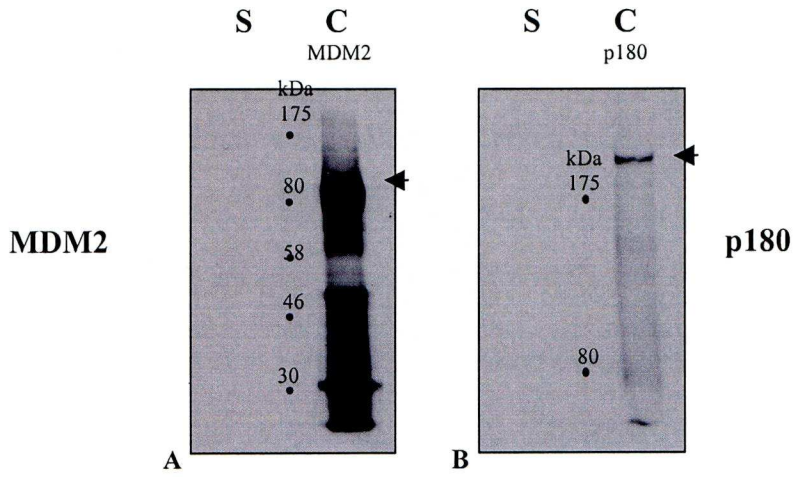


Figure 3.3.6 Western blot analysis of 150mM elution fraction collected during purification process of uninfected insect cells. Presence of MDM2 and p180 was verified in fraction as indicated: **A)** lane marked: S represents sample of 150mM imidazole/elution fraction from uninfected insect cells culture and lane C MDM2 represents MDM2 purified from insect cells in 150mM imidazole/elution fraction which served as a positive control, anti-MDM2 (Ab1, clone IF2) antibody was used for western; **B)** lane marked: S represents sample of 150mM imidazole/elution fraction from uninfected insect cells culture and lane C p180 represents p180 purified from insect cells in 150mM imidazole/elution fraction which served as a positive control, anti-p180 (N-19) antibody was used for western. Main protein bands are indicated by arrows: MDM2 at ~ 90kDa, p180 at ~180kDa.

Results from the purification process, visualized by Brilliant Blue G staining (presented in Figure 3.3.5), suggest that many endogenous insect proteins bind to the Ni-NTA beads and are subsequently eluted with increasing imidazole gradient, with the majority of proteins being eluted in 100mM and 150mM imidazole fractions. The fact that many endogenous insect proteins bind to Ni-NTA beads, and then are eluted in the same condition as MDM2 protein, confirm our concerns about other proteins

being present in MDM2 150mM imidazole elution fraction, which may mediate stimulatory effect on p180 enzymatic activity.

The approximate amount of proteins present in MDM2 150mM imidazole fraction purified from the insect cells and 150mM imidazole fraction obtained from uninfected insect cells, were compared in order to try to determine equivalency for the further assays. Therefore, both samples were separated by SDS-PAGE and stained with Brilliant Blue G (Figure 3.3.7). Based on the staining, it was estimated that the level of background proteins in MDM2 fraction was at least three times greater than in an equivalent volume of the uninfected cells fraction (Figure 3.3.7).

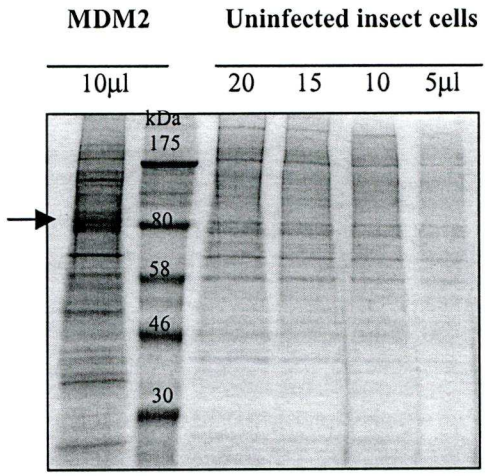


Figure 3.3.7 Brilliant Blue G staining of 150mM imidazole fraction of uninfected insect cells and MDM2 purified from the insect cells. MDM2 from 150 mM imidazole fraction (10µl run on the gel) and 150mM imidazole fraction of uninfected insect cells (different volumes run on the gel from 20 to 5µl) were separated by SDS-PAGE followed by Brilliant Blue G staining in order to compare amounts of background proteins in these fractions. Main MDM2 protein band is indicated by arrow: at ~ 90kDa.

Since many endogenous insect proteins were eluted from uninfected insect cells, but also were present with MDM2 from MDM2 expressing insect cells (Figure 3.3.7) we wanted to examine, prior to the assay with p180, whether any of these fractions displays DNA polymerase activity. Samples were assayed along with p180 and assay buffer for comparison. As presented in Figure 3.3.8, the value obtained for p180 sample was two-fold higher than both the uninfected insect cells or MDM2 fraction, with the latter both being comparable to background value from the assay buffer alone. Based on these observations it was concluded that fractions obtained following the purification procedure from either uninfected insect cells or from MDM2 expressing insect cells do not possess intrinsic DNA polymerase activity.

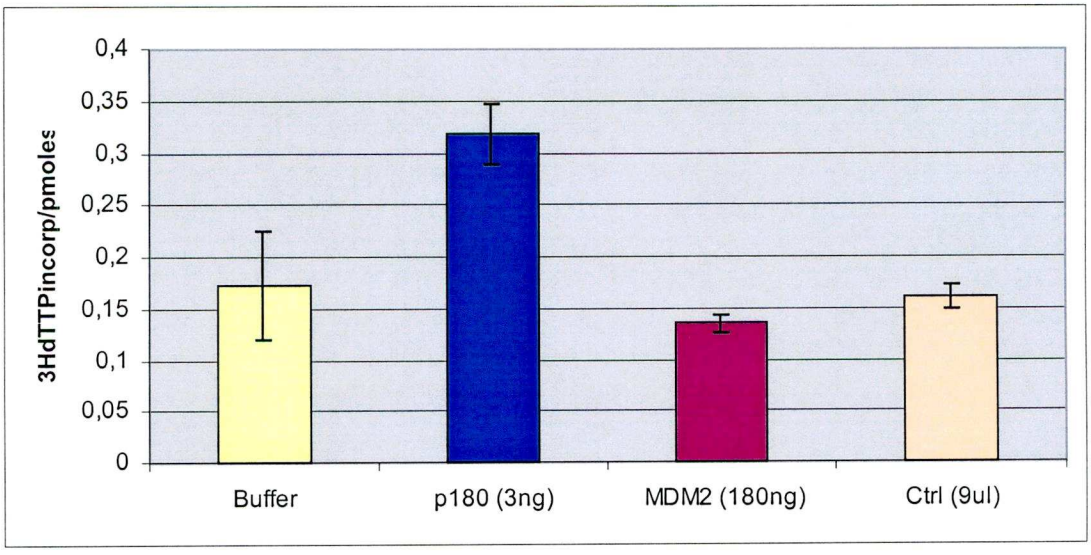


Figure 3.3.8 Examination of intrinsic polymerase activity of MDM2 and uninfected cells fractions. Polymerase activity was measured for the assay buffer (Buffer); 3 ng of p180 - 150 mM imidazole fraction purified from insect cells (p180 (3ng)); 180 ng of MDM2 - 150 mM imidazole fraction purified from insect cells (MDM2 (180ng/3µl)); 9µl of 150 imidazole fraction purified from uninfected insect cells (Ctrl (9µl)). Incorporation of [3H]dTTP (picomoles) was measured by scintillation counting. Data are shown as mean ± standard error of the mean.

Having tested that neither the purified MDM2 fraction nor those from the uninfected insect cells possess intrinsic DNA polymerase activity we examined the effect of purified MDM2 along with fractions of proteins from uninfected insect cells on p180 activity. Since the estimated level of background proteins in the MDM2 fraction was at least three times greater than in an equivalent volume of the uninfected cells fraction, for the DNA polymerase assay, the volume of the control fraction from uninfected insect cells was adjusted to exceed three times the volume of MDM2 fraction, as presented in Table 3.3.3.

Amount of p180	**Amount / volume of MDM2**	**Volume of control fraction** (uninfected insect cells)
3 ng	-	1 µl
	-	3 µl
	-	9 µl
3 ng	60 ng/1 µl	-
	180 ng/3 µl	-
	540 ng/9 µl	-

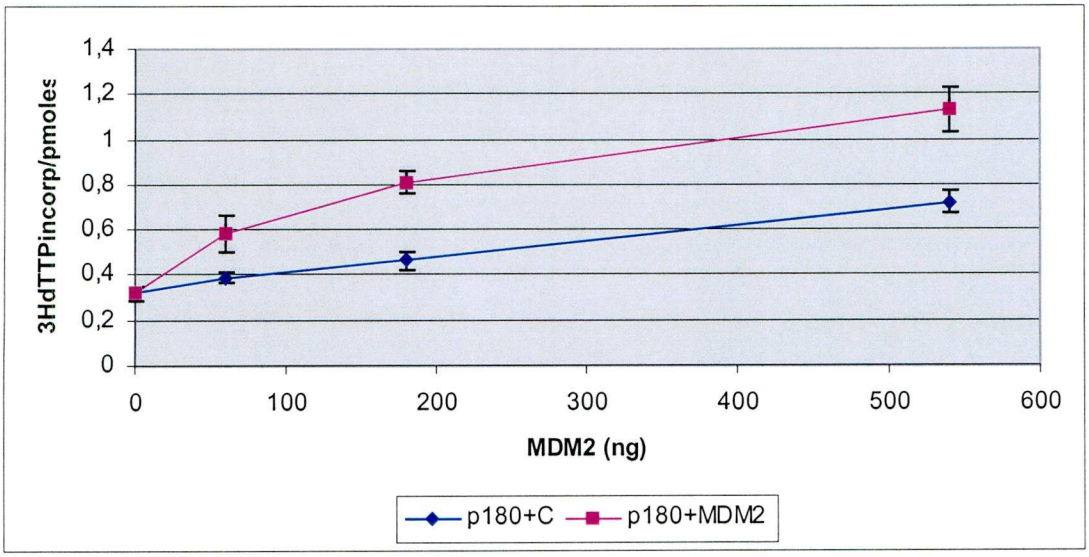
Table 3.3.3 Conditions for DNA polymerase assay with MDM2 purified from insect cells and control uninfected cells. Different amounts/volumes of MDM2 were put into the assay with corresponding amount of background protein/volumes of uninfected insect cells fraction as indicated.

Accordingly, p180 was mixed with varying amounts of either MDM2 or corresponding amounts of control uninfected insect cells fraction e.g. 60ng/1µl of MDM2 was compared with 3µl of control uninfected cells (Table 3.3.3). In addition,

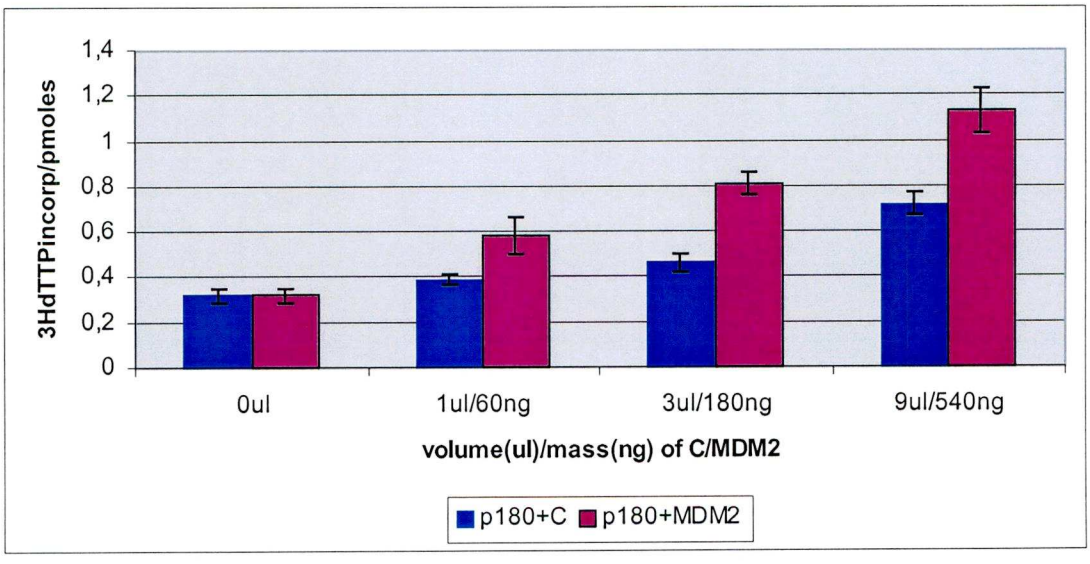
since the quantification of protein in these fractions was based on estimates we also compared equivalent volumes of MDM2 and the control fraction in the assay i.e. 1µl of MDM2 fraction was compared with 1 µl of control uninfected cell fraction. After one hour incubation at 37°C samples were transferred on filters, washed and counted in the liquid scintillation counter. The results using equivalent volumes are presented in Figure 3.3.9 and those from the equivalent amounts of background proteins are presented in Figure 3.3.10.

Comparing activity of p180 upon addition of equal volumes of control from uninfected insect cells and MDM2, it was observed that the activity of p180 was approximately 1.6 fold higher with addition of MDM2 compared to addition of the uninfected cell fraction. However, when these results were analysed taking into account that the volume of the control should exceed the volume of MDM2 by roughly three-fold (in order to have comparable amounts of background proteins), no obvious difference in p180 activity with either MDM2 or uninfected cells fraction was observed, as shown in Figure 3.3.10.

Based on these data it is difficult to conclude whether or not the evidence presented to date demonstrating that MDM2 apparently stimulates p180 activity is a real and specific effect or whether it might be the result of an artifact. It is certainly possible that the purified MDM2 present in the fraction from insect cells is not responsible for stimulatory effect on p180, while other protein/proteins present along with MDM2 or without MDM2 in the fraction from uninfected insect cells may mediate this effect.

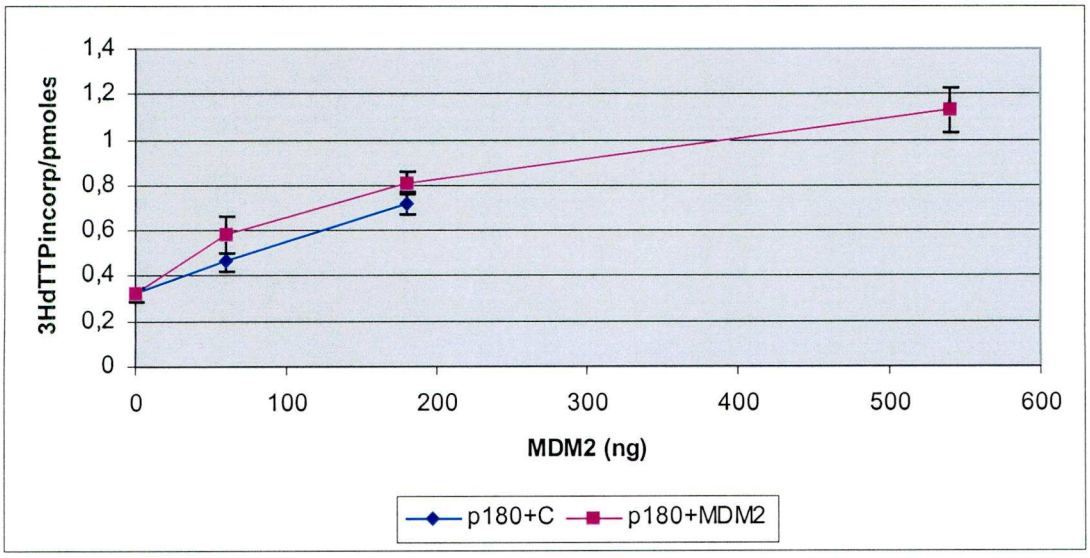


A

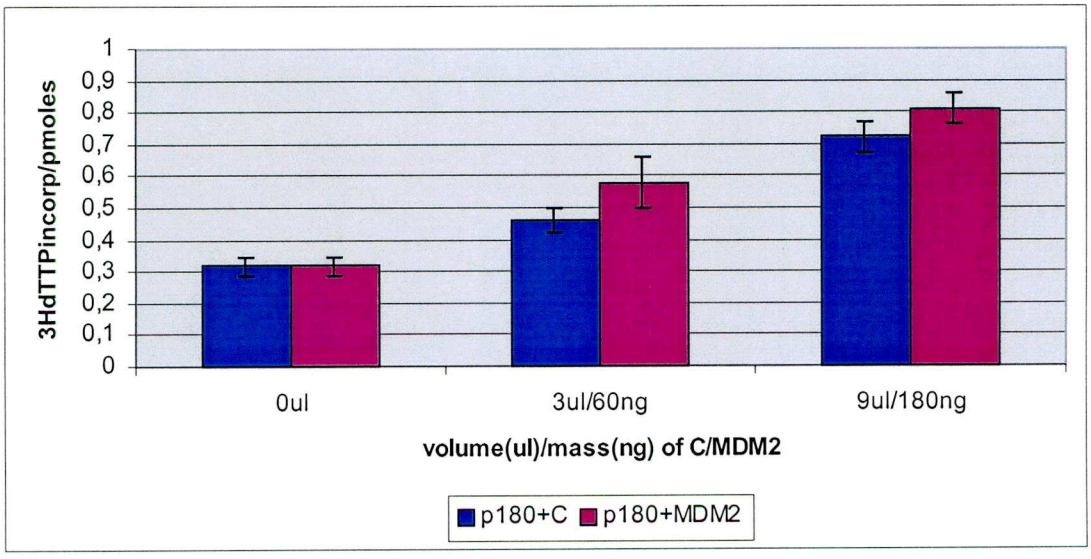


B

Figure 3.3.9 Enzymatic activity of p180 mixed with either control fraction from uninfected insect cells or MDM2. (p180+C) - 3 ng of p180 were assayed with either different volumes of control fraction (1, 3 and 9µl) or (p180+MDM2) different amounts of MDM2 (60ng - 1µl, 180ng - 3µl, 540ng – 9µl). Effects of equivalent volumes of control and MDM2 added to p180 were compared alongside (A – line chart, B – column chart). Data are shown as mean ± standard error of the mean.



A



B

Figure 3.3.10 Enzymatic activity of p180 mixed with either control fraction from uninfected insect cells or MDM2. (p180+C) - 3 ng of p180 were assayed with different volumes of control fraction (3 and 9µl) or (p180+MDM2) - different amounts of MDM2 (60ng - 1µl, 180ng - 3µl). Effects of three times greater volumes of control fraction in comparison to volume of MDM2 added to p180 (A – line chart, B – column chart). Data are shown as mean ± standard error of the mean.

3.3.4 Investigating the specificity of the stimulatory effect of MDM2 on p180 activity: use of MDM2-specific antibodies to try to block the effect

Based on the experimental results presented in section 3.3.3, it seems that MDM2 may not stimulate the polymerase activity of the catalytic subunit of DNA polymerase α (p180), but since the controls for these experiments are imperfect, we therefore wanted to try another approach to investigate this matter.

Therefore, we performed DNA polymerase assays in which MDM2 was added in the presence and absence of various anti-MDM2 antibodies. The idea being to attempt to block MDM2 stimulation by mixing the protein with antibodies mainly recognizing epitopes on its amino – terminal half, which as shown in sections 3.1.3 and 3.1.4 of this thesis is involved in mediating interaction with p180. By using such an approach we predicted two possible outcomes. If an antibody inhibited stimulatory effect of MDM2 on p180 activity, then this would suggest that MDM2 specifically mediates this effect. In the event that no inhibitory effect was observed, the conclusion would be more ambiguous since either the antibody binding to MDM2 has no effect on the ability of MDM2 to stimulate p180 activity or another protein that co-purifies with MDM2 must be responsible for stimulating p180 activity.

Anti-MDM2 antibodies used for the experiment were as follows: Ab1 (clone IF2) – recognizes an epitope within amino acids 26 – 169; SMP14 – raised against amino acids 154 – 167; N-20 – recognizes an epitope at the amino-terminus; in addition anti-MDM2 (C-18), which recognizes an epitope at the carboxy-terminus and anti-p53 (2433) antibodies were used as negative/specificity controls. All the antibodies were mixed with MDM2 and incubated on ice for 15 minutes prior to

adding p180, followed by additional 15 minutes incubation on ice with the enzyme (Table 3.3.4). After one hour incubation at 37°C, samples were transferred to filters, washed and counted in the liquid scintillation counter.

Amount of p180	**Amount of MDM2**	**Antibody used**
3 ng	60 or 180 ng	anti-MDM2 (Ab1) (1μg)
		anti-MDM2 (SMP14) (1μg)
		anti-MDM2 (N-20) (1μg)
		anti-MDM2 (C-18) (1μg)
		anti-p53 (2433) (1μg)

Table 3.3.4 Conditions for DNA polymerase assay with MDM2 purified from insect cells and various antibodies. Varying amounts of MDM2 were added to the assay together with the indicated amounts of antibodies recognising MDM2 or p53 (as a control).

As presented in Figure 3.3.11, the activity of p180 assayed with MDM2 was considerably greater than activity of p180 assayed in the buffer. Upon addition of antibodies, the activity of p180 was not substantially changed. Therefore, as argued above, these results do not shed additional light on the nature of the potential stimulation of p180 activity by MDM2.

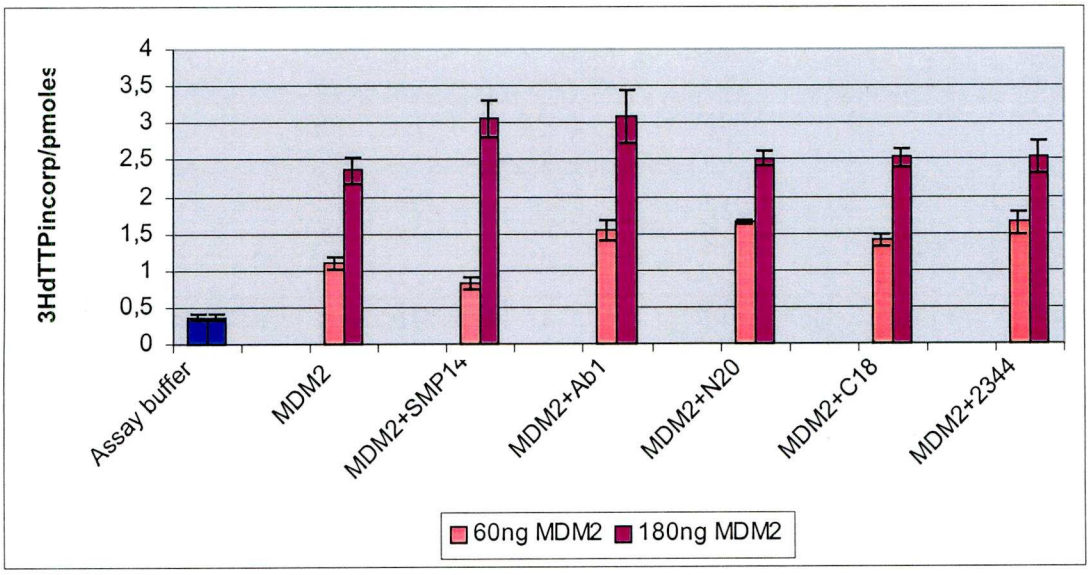


Figure 3.3.11 Enzymatic activity of p180 incubated either with assay buffer, MDM2 or MDM2 pre-incubated with the indicated antibodies. 3 ng of p180 were assayed with the indicated amounts of MDM2 (60 or 180ng) in the presence or absence of indicated antibody. Incorporation of [3H]dTTP (picomoles) was measured by scintillation counting. Data are shown as mean $\pm$ standard error of the mean.

3.3.5 DNA polymerase assay with bacterially expressed GST-MDM2

DNA polymerase assays performed with MDM2 purified from insect cells, revealed that p180 activity was stimulated, but we could not determine whether or not this was due to the presence of other protein/proteins in addition to the MDM2 added to the assay (section 3.3.3). To try to resolve this uncertainty we have pursued another approach in which MDM2 is produced in a heterologous system. We chose to test the effect of MDM2 expressed in bacterial system for a number of reasons. Firstly, in other studies (134) we had found that expression and purification of

glutathione S-transferase (GST) tagged MDM2 protein allowed us to obtain relatively large amounts of fairly pure MDM2 and moreover, this would probably be contaminated with a different set of contaminant proteins than were present in the insect cells. Perhaps more significant, MDM2 purified in this manner retains its E3 ligase activity for up to 24 hours (134), and this can be verified by performing *in vitro* ubiquitylation assays. By using freshly prepared GST-MDM2 versus GST-MDM2 protein from frozen aliquots, it would be possible for us to compare an effect of enzymatically active and inactive MDM2 on p180 polymerase activity.

3.3.5.1 DNA polymerase assay with frozen (enzymatically inactive) aliquots of bacterial GST-MDM2

In order to perform DNA polymerase assays with GST-MDM2, the protein was purified from *Escherichia coli,* as described in Materials and methods (section 2.11). Since glutathione S-transferase (GST), used to facilitate purfication of MDM2 can be readily purified in the absence of a fusion partner, we could use purified GST as an appropriate control. For these experiments the purified GST was purified by a former member of our research group Dr. Tim Devling. In order to estimate the amount of GST-MDM2 and GST present in elution fractions, samples were subjected to SDS-PAGE followed by Brilliant Blue G staining with BSA titrated providing a standard (Figure 3.3.12). Based on the Brilliant Blue G staining, it was estimated that elution fractions contained approximately 20 ng per µl of GST-MDM2, whereas the elution fraction of GST approximately ten times more protein (200ng/µl).

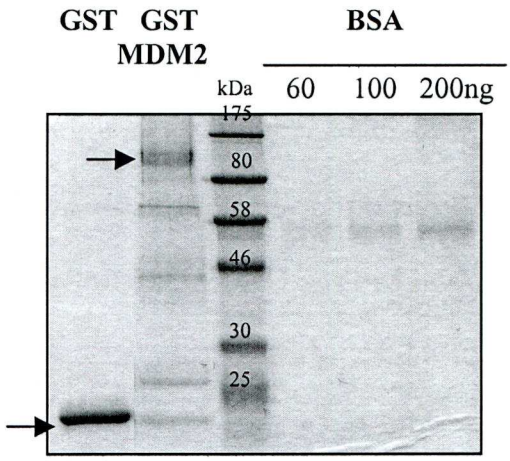


Figure 3.3.12 Brilliant Blue G staining of GST and GST-MDM2 proteins purified from *E.coli*. GST and GST-MDM2 proteins purified from *E.coli* were separated by SDS-PAGE followed by Brilliant Blue G staining, BSA was used as a protein amount standard. Main protein band are indicated by arrows: GST at ~ 26kDa, GSTMDM2 at ~ 120kDa.

In the first instance we tested whether excess GST-MDM2 could stimulate a range of p180 concentrations with GST as a control normalized either for matched volume or protein concentration (based upon our earlier estimates) (Table 3.3.5)

Amount of p180	**Amount of GST-MDM2**	**Amount of GST**
3ng	-	180ng or 1.8µg/9 µl
10ng		
20ng		
3ng	180ng/9µl	-
10ng		
20ng		

Table 3.3.5 Conditions for DNA polymerase assay with GST-MDM2 and GST purified from *E.coli*. Different amounts of p180 were put into the assay with GST-MDM2 and GST as a control as indicated.

Based on the results presented in Figure 3.3.13 it can be observed that neither GST nor GST-MDM2 possess intrinsic polymerase activity. Moreover, the activity of p180 was not altered upon addition of either GST-MDM2 or GST as Figure 3.3.13 illustarates.

GST-MDM2 purified from *E.coli* and stored frozen in –80°C does not stimulate the activity of DNA polymerase α catalytic subunit. These findings support earlier observations suggesting that the stimulatory effect on p180 polymerase activity was not mediated by MDM2 protein (Figure 3.3.10).

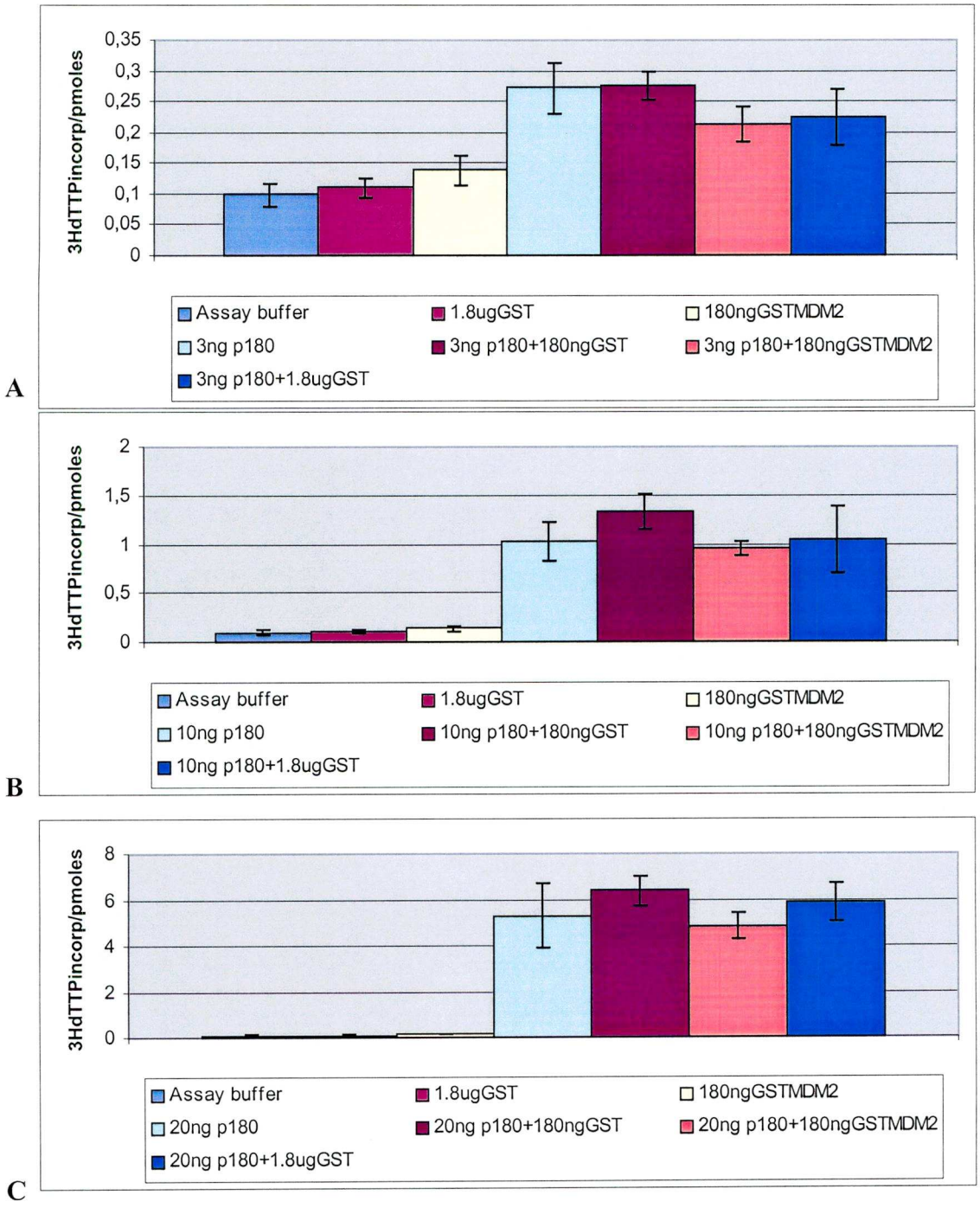


Figure 3.3.13 Enzymatic activity of p180 assayed with enzymatically inactive MDM2. Different amounts of p180 (**A**-3ng, **B**-10ng, **C**-20ng) mixed with either assay buffer, GST or GST-MDM2 (frozen batch) purified from *E.coli*. Incorporation of [3H]dTTP (picomoles) was measured by scintillation counting. Data are shown as mean ± standard error of the mean.

3.3.5.2 Assay of DNA polymerase α activity in the presence of freshly purified (i.e. E3 ligase active) GST-MDM2

MDM2 possesses an E3 ligase activity (64) that might alter DNA polymerase α activity and which is lost when the enzyme is stored frozen or within 24 hours when stored at 4°C. We therefore decided to test freshly prepared GST-MDM2 protein, which retain its E3 ligase activity, in order to examine whether enzymatically active GST-MDM2 may alter p180 activity.

Both GST and GST-MDM2 were purified from *E.coli* and immediately after purification proteins were subjected to SDS-PAGE followed by staining with Brilliant Blue G in order to confirm the presence and estimate the yield of proteins of interest (Figure 3.3.14). Based on the Brilliant Blue G staining, compared to a BSA standard, it was estimated that elution fractions contains approximately 20 ng per µl of GST-MDM2, whereas elution fraction of GST yielded approximately ten times more protein (200ng/µl).

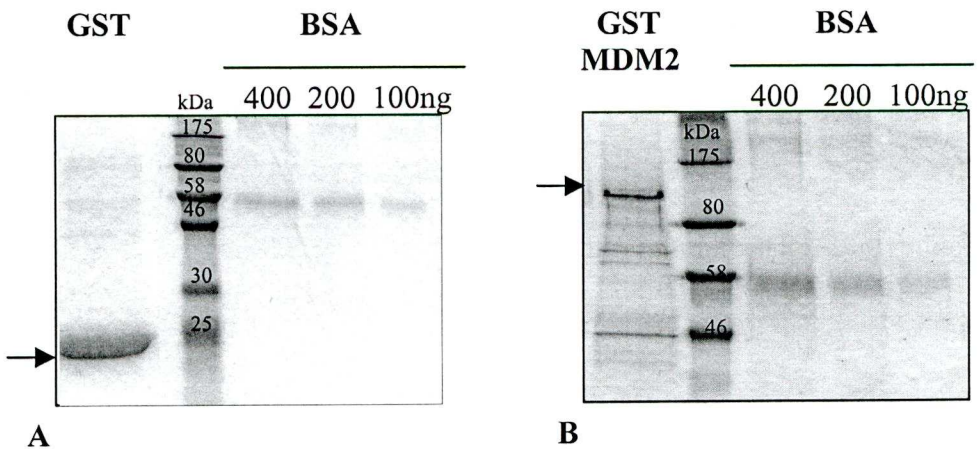


Figure 3.3.14 Brilliant Blue G staining of purified GST and GST-MDM2 proteins. GST (A) and GST-MDM2 (B) were separated by SDS-PAGE followed by Brilliant Blue G staining. BSA was used as a protein amount standard. Main protein band are indicated by arrows: GST at ~ 26kDa, GST-MDM2 at ~ 120kDa.

In vitro ubiquitylation assays were performed to test whether purified GST-MDM2 was able to mediate auto-ubiquitylation *in vitro*, thus confirming whether MDM2 retained its E3 ligase activity. GST-MDM2 was incubated in the presence and absence of ubiquitin (Ub) at 30°C overnight. After incubation samples were subjected to SDS-PAGE and western blotting (Figure 3.3.15). As shown in Figure 3.3.15, in the presence of ubiquitin, purified GST-MDM2 catalyzed an auto-ubiquitylation reaction, which confirmed its enzymatic activity.

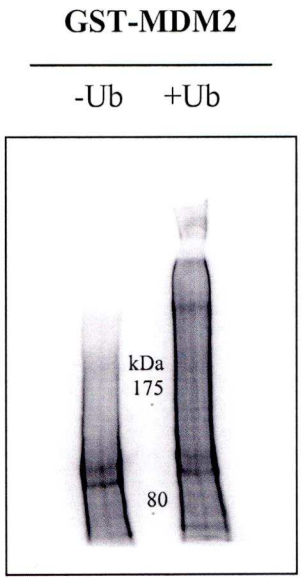


Figure 3.3.15 GST-MDM2 *in vitro* ubiquitylation assay. In the presence of ubiquitin (Ub) GST-MDM2 catalyzes autoubiquitylation reaction *in vitro*. Samples were subject to SDS-PAGE followed by western blotting with anti-MDM2 antibody (Ab1, clone IF2).

For the DNA polymerase assay p180 was titrated with either 180 ng of GST-MDM2 or GST, or 1.8 µg of GST thus comparing either equal estimated protein concentrations or eluate volumes respectively, as presented in Table 3.3.5.

Based on the results presented in Figure 3.3.16 it can be observed that neither GST nor GST-MDM2 possess intrinsic polymerase activity. Moreover, the activity of p180 was not altered upon addition of either enzymatically active GST-MDM2 or GST as presented in Figure 3.3.16.

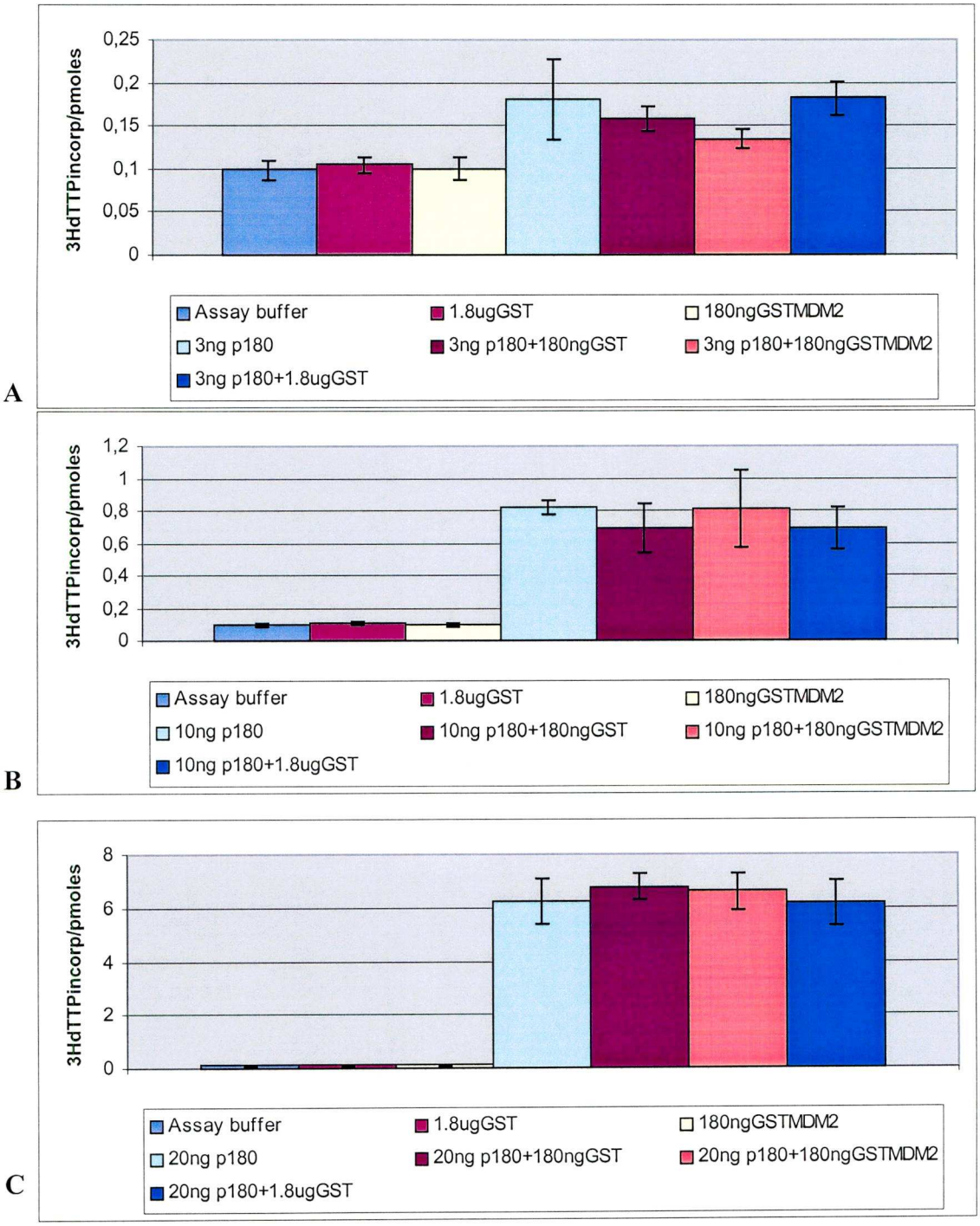


Figure 3.3.16 Enzymatic activity of p180 assayed with enzymatically active MDM2. Enzymatic activity of different amounts of p180 (**A**-3ng, **B**-10ng, **C**-20ng) mixed with either assay buffer, GST or freshly purified GST-MDM2 from *E.coli*. Incorporation of [3H]dTTP (picomoles) was measured by scintillation counting. Data are shown as mean ± standard error of the mean.

In summary, results presented on the involvement of MDM2 in altering p180 enzymatic activity do not provide clear evidence for such an effect, since the activity of DNA polymerase α was not altered upon addition of bacterially purified GST-MDM2 either enzymatically active or inactive, whilst results from assays with MDM2 purified from insect cells seem to be ambiguous. In the latter experiments, in which we observed stimulation of polymerase activity of p180 upon addition of MDM2-enriched fractions, our main concern was the purity of used MDM2 protein, and subsequent experiments with equivalent fractions from uninfected insect cells provided further support for this concern. Hence, to overcome the problem of impure MDM2, we collaborated with Stuart Linn at U.C. Berkley, who has shown previously a stimulatory effect of MDM2 on DNA polymerase ε activity, where exceptionally pure MDM2 was used (59). To examine MDM2 effect on polymerase activity of DNA polymerase α, an assay with MDM2 expressed in insect cells (59) and DNA polymerase α holoenzyme purified from HeLa cells was performed. As observed in Figure 3.3.17A polymerase activity of DNA polymerase α was increased substantially upon addition of MDM2, whilst this effect of MDM2 was inhibited when anti-DNA polymerase α antibody was added (Figure 3.3.17B). Hence, these findings provide more convincing though not unequivocal evidence that DNA polymerase α activity is stimulated by MDM2.

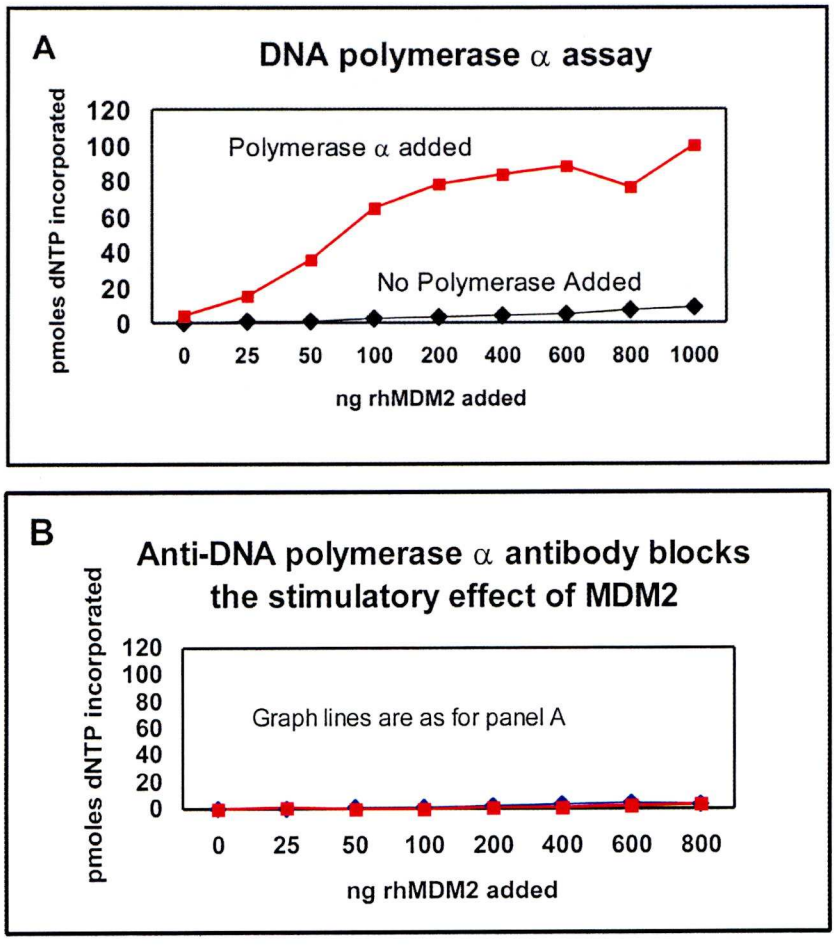


Figure 3.3.17 Enzymatic activity of DNA polymerase α holoenzyme purified from HeLa cells. A) MDM2 purified from insect cells (59); **B)** anti-DNA polymerase α antibody (experiment was performed by Prof. S. Linn).

3.4 Examination of ability of MDM2 to mediate modifications of p180

Results presented in this thesis thus far indicate that MDM2 interacts with p180 both *in vitro* and *in vivo*. In addition, work from our collaboration with Stuart Linn at U.C. Berkley suggests that MDM2 may stimulate the catalytic activity of the DNA polymerase α holoenzyme (as Figure 3.3.17 shows). Since many MDM2 interacting proteins are subject to ubiquitylation (and often therefore to subsequent degradation in proteasomes) mediated by MDM2 E3 ligase activity (64,151), we decided to test whether MDM2 might also modify p180. To the best of our knowledge, there are no reports linking p180 with the ubiquitin/proteasome pathway. Therefore, we first wanted to examine whether inhibition of the 26S proteasome complex might have an effect on p180 levels in cells and might therefore suggest, that p180 was subject to proteasomal degradation with the possibility that MDM2 might be involved in this.

3.4.1 MG132 addition to MCF-7, H1299 and Clone 9 cells

In order to test whether p180 levels were changed upon addition of a well known inhibitor of 26S proteasomes - MG132, the inhibitor was applied to MCF-7, H1299 and Clone 9 (H1299) cells in culture. H1299 and Clone 9 cells are routinely grown in our laboratory, (Clone 9 cells are derived from H1299 cells but express high levels of MDM2 since the cells were stably transfected with pCMVNeoBamMdm2) and do not express p53; whilst MCF-7 cells were included in the experiment because Dr. Claire Gibney was testing the effect of MG132 on MCF-7 cells as part of a different study and kindly provided samples used in this

experiment. Cells were treated with 100μM MG132 and incubated for 0, 2, 4, and 6 hours before harvesting and then being subjected to SDS-PAGE and analysed by western blotting.

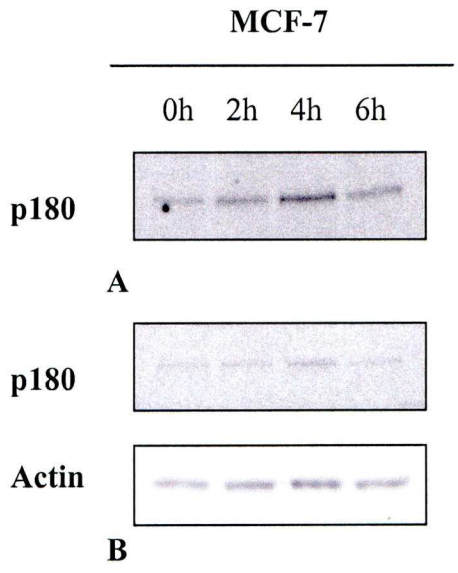


Figure 3.4.1 Effect of MG132 addition to MCF-7 cells. MCF-7 cells in culture were treated with MG132 to a final concentration of 100μM and harvested by trypsinization at indicated time points: 0, 2, 4 and 6 hours; 50μg of total protein, from each sample was subjected to SDS-PAGE followed by western blot using anti-p180 (N-19) and anti-actin (C-2) antibodies as indicated; **A)** western blot analysis performed on the samples separated on 6% polyacrylamide gel; **B)** western blot on samples separated on 10% polyacrylamide gel (samples were provided by Dr. C. Gibney).

In Figure 3.4.1, we can see that the level of p180 after 4 hours of treatment with MG132 is considerably higher than for 0, 2 or even 6 hours treatment. However, the sample loading (indicated by the actin level) may be slightly higher for this lane (Figure 3.4.1B, lower panel).

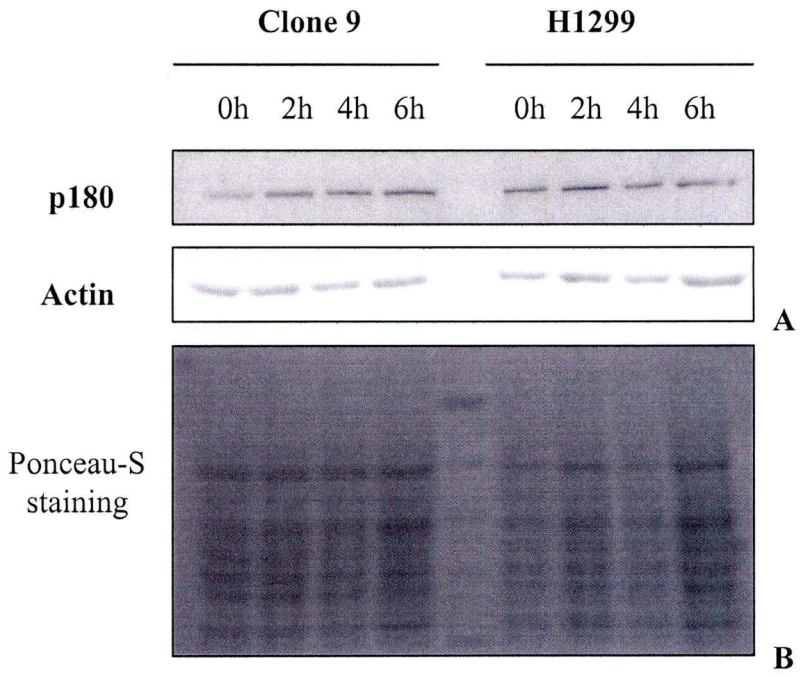


Figure 3.4.2 Effect of MG132 addition to Clone 9 and H1299 cells. Cells in culture were treated with MG132 to a final concentration of 100μM and harvested by trypsinization at the indicated time points; 50μg of total protein, from each sample was analyzed by SDS-PAGE followed by western blot; **A)** western blot with anti-p180 (N-19) and anti-actin (C-2) antibodies as indicated; **B)** Ponceau S staining of the blot as an indicator of samples loading.

The results for Clone 9 partly confirmed results for MCF-7 cells with stabilization of p180 being observed after treatment for 2, 4 and 6 hours with MG132 (Figure 3.4.2A). In this case sample loading appears equal as indicated by actin levels although Ponceau S staining suggests that the 6h time point sample was overloaded (Figure 3.4.2B).

Based on the western blot analysis of samples from H1299 cells treated with MG132, it was not obvious whether stabilization of p180 occurred (Figure 3.4.2A). It appeared that loading was not equal, samples for time points 2 and 6 hours were

overloaded (Figure 3.4.2A-actin, 3.4.2B). Nevertheless, the levels of p180 were not considerably higher for these two time points suggesting that stabilization did not take place.

In summary, results obtained for MCF-7 and Clone 9 cells, suggested that p180 might be subject to regulation by the ubiquitin/proteasome pathway, whilst this was not observed in H1299 cells expressing relatively low levels of MDM2, especially when compared to Clone 9 cells, which might suggest involvement of MDM2 in that process. However, in general data presented do not provide strong evidence for p180 being subject to ubiquitin/proteasome degradation pathway, especially in the light of findings presented in report from Wong SW *et al.* 1986 (109) which shows no evidence for intracellular degradation of p180, and estimates half life of ≥15 hours for p180. Despite this we were determined to examine p180 ubiquitylation in cells, encouraged by the findings that proteins involved in DNA replication such as Cdc45 or PCNA are subject of that modification as well (197,198). Moreover, we still wanted to test the potential involvement of MDM2 in mediating modification of p180, since MDM2 mediates ubiquitylation of many of its interacting proteins (reviewed in 151). Therefore, we decided to utilize an *in vivo* ubiquitylation assay which enables us to address these issues.

3.4.2 *In vivo* ubiquitylation assay to examine MDM2 involvement in mediating p180 modification

To examine whether MDM2 might modify p180 by ubiquiltylating it we performed an *in vivo* ubiquitylation assay based upon purification of ubiquitin-conjugates by affinity chromatography. The *in vivo* ubiquitylation assay we used

enables identification of substrates that are covalently modified by addition of 6xHis-tagged ubiquitin moieties expressed in the cell that can be purified by binding of these to Ni-NTA agarose beads.

H1299 cells were transfected with constructs expressing variously 6xHis-tagged ubiquitin, MDM2 and/or p180, as described in Figure 3.4.3 lanes 2-8. We wanted to examine whether p180 could be pulled down (i.e. affinity purified) along with 6xHis-tagged ubiquitin, in either the absence or presence of transiently transfected MDM2 (Figure 3.4.3 lanes 3 and 5 respectively). An increase in the amount of p180 affinity purified in the presence of transfected MDM2 would suggest MDM2 involvement in p180 ubiquitylation. In addition, cells transfected solely with 6xHis-tagged ubiquitin provided an indication of endogenous ubiquitylation levels of the proteins of interest (Figure 3.4.3 lane 2). Lanes 1, 4, 6 and 8 serve as specificity controls for the assay, since 6xHis-tagged ubiquitin was not transfected and any proteins pulled down are the result of non-specific interactions of these proteins with Ni-NTA agarose beads. Finally, the samples in lanes 5 and 7 from cells transfected with MDM2 and 6xHis-tagged ubiquitin which should (and do) display detectable MDM2 E3 ligase activity, and thus act as a positive controls for the assay (Figure 3.4.3).

Transfected cells were processed as described in Materials and methods (section 2.14), and harvested after 48 hours and then divided into two aliquots: one, aliquot consisting of 10% of harvested cells was used for western blot analysis, a second aliquot consisting of 90% of the sample was processed for the *in vivo* ubiquitylation assay. In order to purify proteins conjugated to 6xHis-tagged ubiquitin, lysates from 90% aliquots were incubated with Ni-NTA beads for 4 hours,

washed and eluted with sample buffer supplemented with 200mM imidazole. Eluted samples along with cells lysates were subjected to SDS-PAGE followed by western blot analysis with antibodies against p180 (N-19) and MDM2 (Ab1, clone IF2) (Figure 3.4.3).

Results presented in Figure 3.4.3 show, as expected, that MDM2 is detected as ubiquitylated in the presence of 6xHis-tagged ubiquitin and un-surprisingly this was unaffected by co-transfection of p180 (lanes 5 and 7). Although, the signal obtained for p180 in lanes 3 and 5 seems to be the strongest, it is difficult to assess whether this is an indication of p180 ubiquitylation since a slightly weaker band is also present in lane 4 which suggests that p180 binds non-specifically to the Ni-NTA beads. It is known that p180 is capable of binding to metal ions such as Mn++ or Mg++ during catalysis (199), in addition p180 possesses a Zn finger motif (9) and this may form the basis for the observed result.

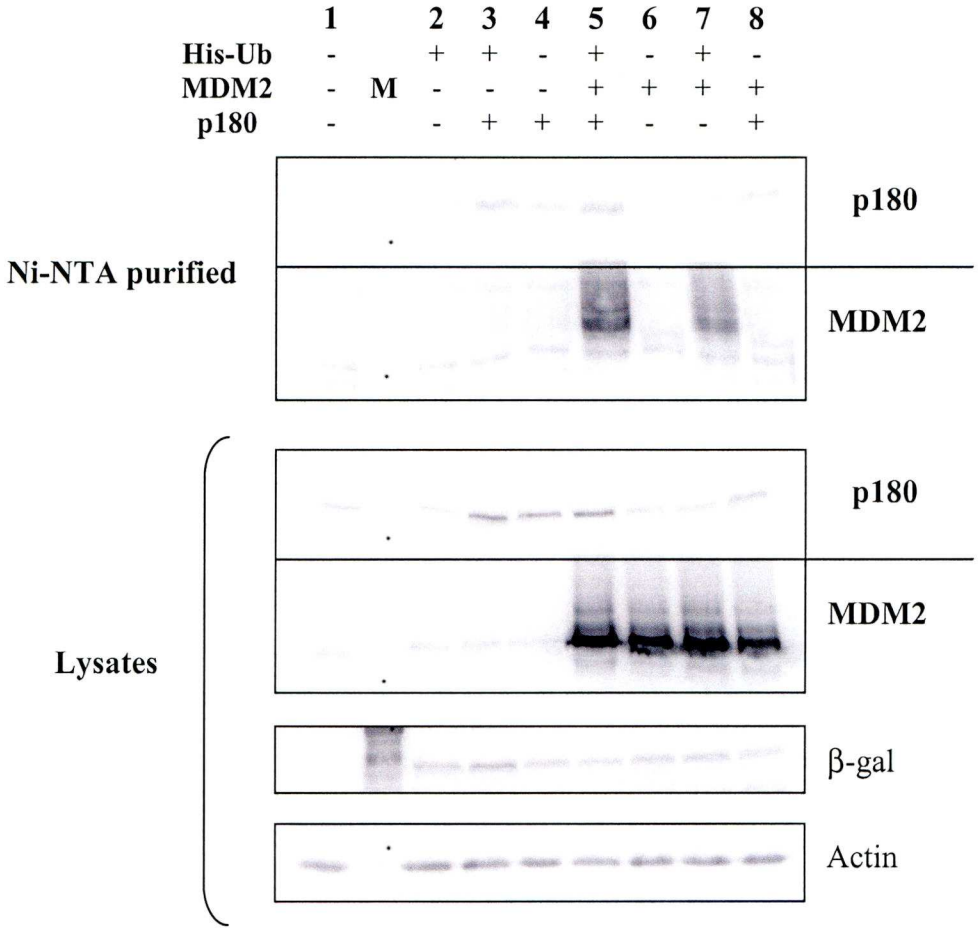


Figure 3.4.3 *In vivo* ubiquitylation assay with 6xHis-tagged ubiquitin, p180 and MDM2. H1299 cells were transfected 6xHis-tagged ubiquitin (15 μg), p180 (30 μg) and MDM2 (30 μg) transfected using GeneJuice™ reagent in several combinations, as indicated. Ni-NTA purified section (upper panels as indicated) presents data for proteins purified with 6xHis-tagged ubiquitin on Ni-NTA agarose beads; Lysates section (lower panels as indicated) illustrate the steady-state levels of the indicated proteins with or without transfection. Note that all transfected samples included a plasmid that expresses β-galactosidase (6μg) to enable comparison of transfection efficiency and also actin provides loading information. Western blots were performed with anti -p180 (N-19), -MDM2 (Ab1, clone IF2), -actin (C-2) and -β-galactosidase (Ab1, clone 200-193) antibodies.

Despite these issues with binding of p180 to Ni-NTA beads, we attempted to perform another assay to clarify whether p180 might be ubiquitylated and also determine whether MDM2 is engaged in that process. A RING finger mutant of MDM2 (Cys464Ala), which lacks the ability to mediate ubiquitylation and a mutant of 6xHis-tagged ubiquitin (Lys29,48,63Arg) incapable of forming ubiquitin chains were included in the experiment. By using the RING finger mutant we were hoping to overcome potential endogenous effect of wild type MDM2 promoting p180 ubiquitylation (as suggested by lane 3 in Figure 3.4.3), while 6xHis-tagged ubiquitin (Lys29,48,63Arg) was used in order to detect only monoubiquitylation of p180.

H1299 cells were transfected with combinations of p180, MDM2, MDM2 RING finger mutant, 6xHis-tagged ubiquitin and 6xHis-tagged ubiquitin mutant, as described in Figure 3.4.4 lanes 2-7. Transfected cells were then processed as described in Materials and methods (section 2.14) and proteins purified on Ni-NTA beads, along with cells' lysates were subjected to SDS-PAGE analysis followed by western blot (Figure 3.4.4).

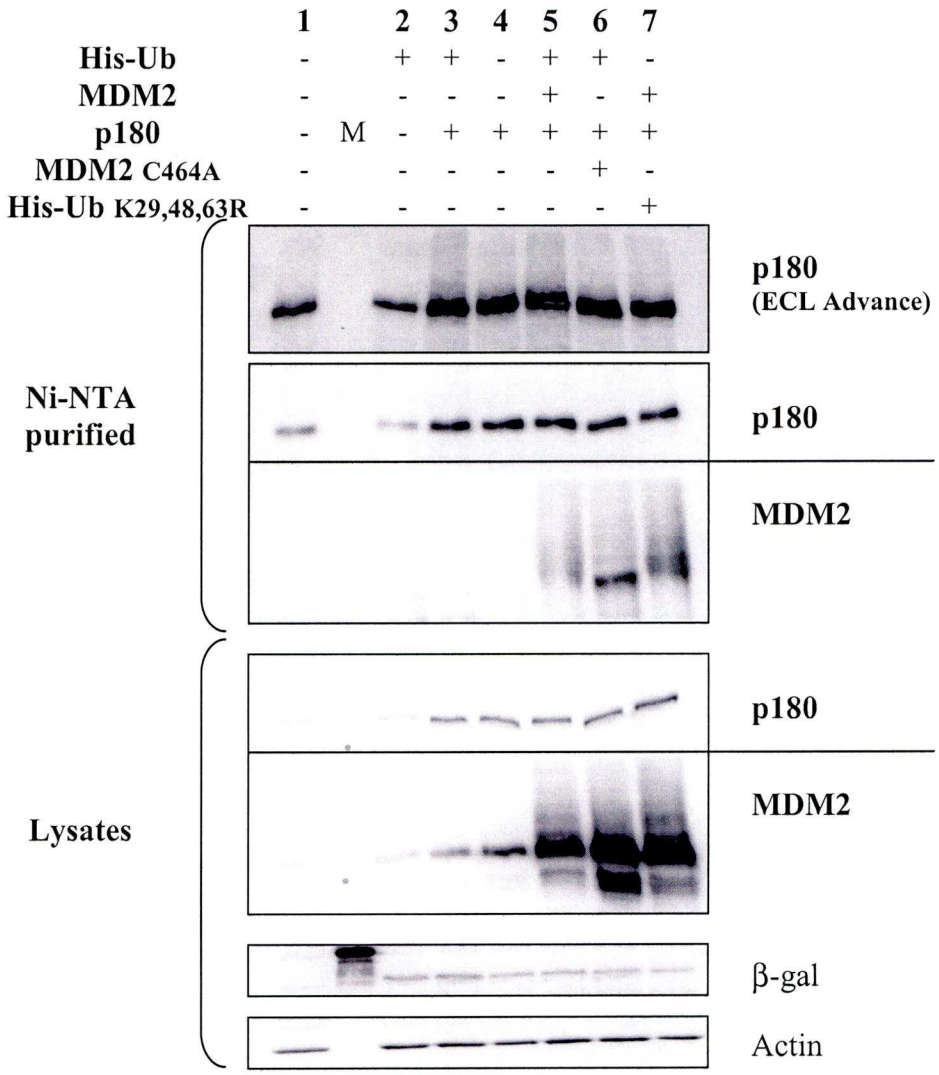


Figure 3.4.4 *In vivo* ubiquitylation assay with 6xHis-tagged ubiquitin, 6xHis-tagged mutant ubiquitin (K29,48,63R), p180, MDM2 and mutant MDM2 (C464A). H1299 cells were transfected with 6xHis-tagged ubiquitin (15 μg), 6xHis-tagged mutant ubiquitin (K29,48,63R) (15 μg), p180 (30 μg), MDM2 (30 μg) and mutant MDM2 (C464A) (30 μg) using GeneJuice™ reagent in several combinations, as indicated. Ni-NTA purified section presents proteins purified with 6xHis-tagged ubiquitin on Ni-NTA agarose beads; Lysates section show effect of cell's transfection, with β-galactosidase (6μg) as an transfection efficiency control and actin as an loading control. Western blot was performed with anti -p180 (N-19), -MDM2 (Ab1, clone IF2), -actin (C-2) and -β-galactosidase (Ab1, clone 200-193) antibodies. ECL Advance Western Blotting Detection System was used in the indicated panels to provide greater sensitivity (GE Healthcare).

As expected, MDM2 was ubiquitylated in the presence of 6xHis-tagged ubiquitin (lane 5) and also MDM2 ubiquitylation was inhibited in cells transfected with an MDM2 RING finger mutant (lane 6). In addition, MDM2 was detectably monoubiquitylated in the presence of 6xHis-tagged ubiquitin mutant (Lys29,48,63Arg) (lane 7). In order to enhance the signal obtained for Ni-NTA purified p180 we used the ECL™ Advance Western Blotting Detection System (GE Healthcare Life Sciences) (Figure 3.4.4, ECL Advance panel). Potentially of significance, the p180 signal observed in lanes 3 and 5 displayed an increased high molecular weight smear and this was independent of MDM2 transfection, which might be indicative of multiple-mono- or poly-ubiquitylation of p180. However, bands corresponding to the main (presumably mono- or unmodified) p180 were also present in other lanes of the Ni-NTA section (Figure 3.4.4, lanes 3-7) whenever p180 was transfected which undermines any such conclusion (Figures 3.4.3-4).

Since the previous experiments were significantly compromised by the binding of p180 to Ni-NTA beads another approach was tested. To examine whether p180 was ubiquitylated in the cells and whether MDM2 was involved in that process, cells were transfected with HA-tagged ubiquitin, MDM2 and p180 in different combinations (Figure 3.4.5 lanes 1-5) and an antibody that recognizes the HA tag was used to immunoprecipitate protein complexes from cell lysates. Immunoprecipitation was carried out as described in Materials and methods (section 2.10), and samples were then separated by SDS-PAGE and analysed by western blotting. No bands corresponding to p180 could be detected in the IP panel, which suggests that p180 was not conjugated to ubiquitin *in vivo* (Figure 3.4.5, p180 IP

panel). As expected MDM2 was detected in lanes 3 and 4 which is indicative of MDM2 ubiquitylation.

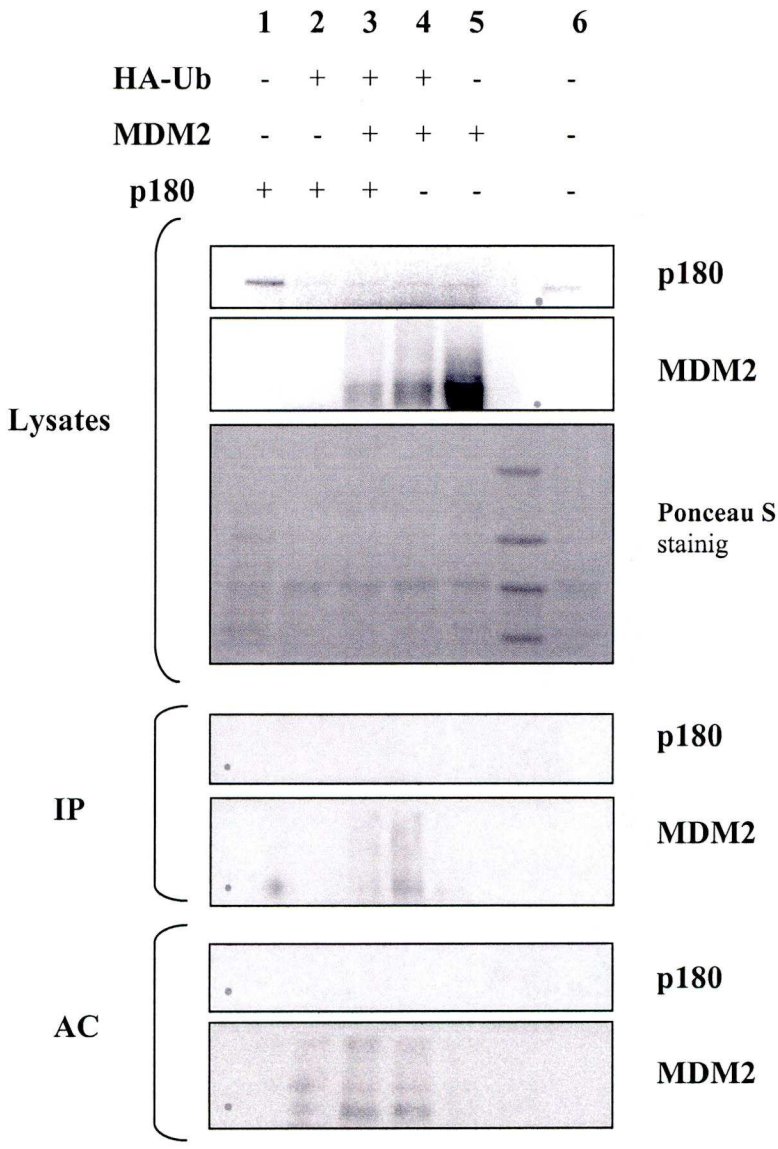


Figure 3.4.5 Co-immunoprecipitation experiment with HA-tagged ubiquitin, p180 and MDM2. H1299 cells were transfected with HA-tagged ubiquitin (30 μg), p180 (30 μg) and MDM2 (30 μg) using GeneJuice™ reagent in several combinations, as indicated. Immunoprecipitation was performed on 3.5mg of total protein with anti-HA (16B12) antibody (IP panel) and anti-CD20 (Leu16) as an antibody control (AC panel). For western blot analysis anti-p180 (N-19) and anti-MDM2 (Ab1, clone IF2) antibodies were used; Ponceau-S staining served as a loading control.

In the light of presented results (Figure 3.4.3-5), there is no clear evidence for p180 ubiquitylation in the cells studied. Since in immunoprecipitation experiments MDM2 was shown to form a complex with DNA polymerase α in cells, there is a possibility that apart from interacting with catalytic subunit of DNA polymerase α (p180) MDM2 might interact with other subunits of this heterotetrameric enzyme complex. This notion seems to be further supported by immunoprecipitation data with MDM2 mutants either consisting of the amino-terminal 166 amino acids or lacking this region. Whilst MDM2 was shown to interact directly with p180, where the amino-terminal 166 amino acids of MDM2 appeared to be required for that interaction *in vitro*, on the contrary for complex formation in cells this region was dispensable. Hence, it appeared that the first 166 amino acids of MDM2 interact directly with p180, whereas the remaining region could be involved in interaction with other subunits of DNA polymerase α in cells explaining complex formation between DNA polymerase α and MDM2 mutants lacking the amino-terminal 166 amino acids. The possibility of this potential MDM2 interaction with other subunits of DNA polymerase α encouraged us to perform another *in vivo* ubiquitylation assay in order to examine modification of both primase subunits of DNA polymerase α.

H1299 cells were transfected with combinations of p180, MDM2 and 6xHis-tagged ubiquitin as described in Figure 3.4.6. Transfected cells were processed as described in Materials and methods (section 2.14), and harvested after 48 hours, however on this occasion either 10μM of MG132 (in DMSO) was added to cells four hours before harvest in order to block proteasome dependent degradation and thus increase the amount of ubiquitylated proteins in the cells, or DMSO to test the effect of the drug vehicle on cells. During harvest cells were divided into two aliquots: one,

aliquot consisting of 10% of harvested cells was used for western blot analysis, a second aliquot consisting of 90% of the sample was processed for the *in vivo* ubiquitylation assay. In order to purify proteins conjugated to 6xHis-tagged ubiquitin, lysates from 90% aliquots were incubated with Ni-NTA beads for 4 hours, washed and eluted with sample buffer supplemented with 200mM imidazole. Eluted samples along with cells lysates were subjected to SDS-PAGE followed by western blot analysis with antibodies against 48kDa primase subunit (PRIM1), 58kDa second primase subunit (PRIM2), p180 (N-19) and MDM2 (Ab1, clone IF2) (Figure 3.4.6).

We failed to address the main question, whether primase subunits were ubiquitylated, since we were unable to detect both subunits either in the lysates nor Ni-NTA purified probably due to problems with antibodies (Figure 3.4.6). However, we observed that in the cells treated with MG132, p180 appeared to be affinity purified with ubiquitin (lane 7), while the amount of p180 purified with 6xHis-tagged ubiquitin seems to be diminished upon co-transfection with MDM2 (lane 8). Contrary to results presented in previous experiments (see Figure 3.4.3-5) we can observe that p180 might be indeed subject to ubiquitylation in cells, whilst MDM2 appears to have an inhibitory effect on mediating that p180 modification. However, these findings need to be further tested since the results presented here are an example of a single experiment performed at the end of the time available for this project.

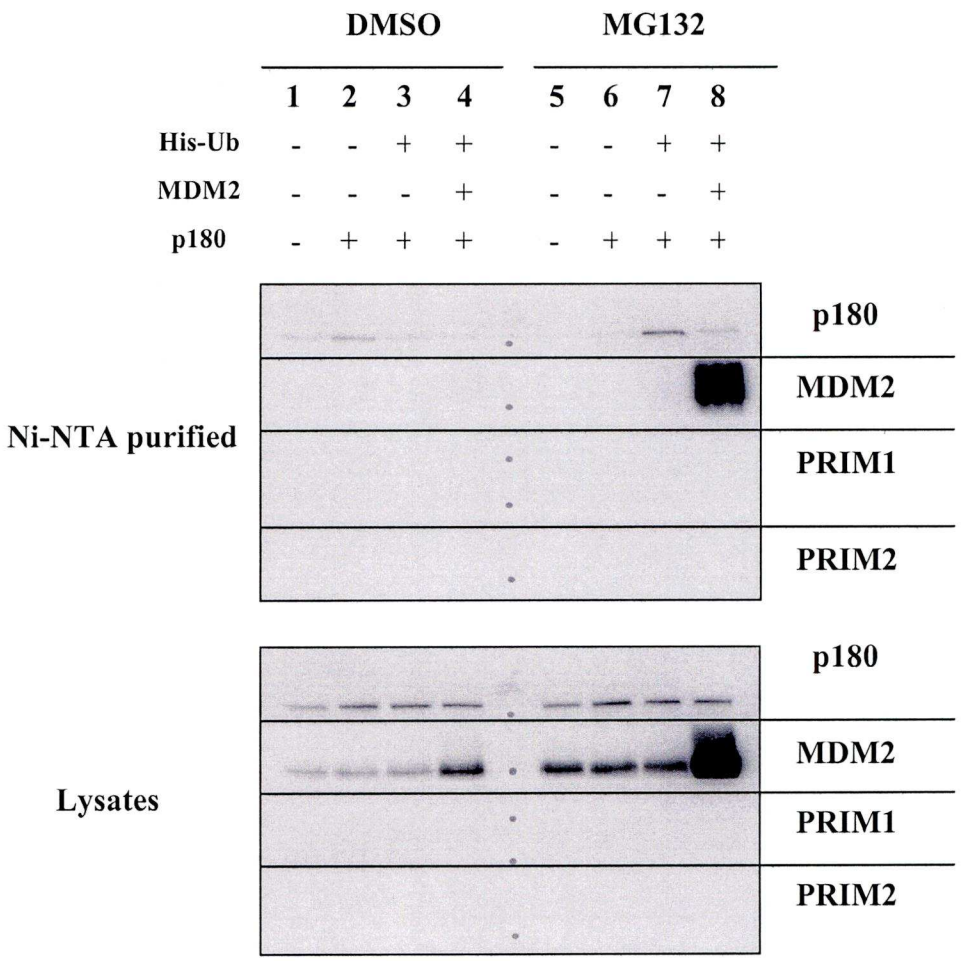


Figure 3.4.6 *In vivo* ubiquitylation assay with 6xHis-tagged ubiquitin, p180 and MDM2 with addition of MG132. Cells were transfected with 6xHis-tagged ubiquitin (15 μg), p180 (30 μg) and MDM2 (30 μg) using PEI (polyethylenimine) reagent at a 1:1 w:v ratio to DNA, in several combinations as indicated. Ni-NTA purified section (upper panels as indicated) presents data for proteins purified with 6xHis-tagged ubiquitin on Ni-NTA agarose beads; Lysates section (lower panels as indicated) illustrate the steady-state levels of the indicated proteins with or without transfection. Note that all transfected samples included a plasmid that expresses β-galactosidase (6μg) to enable comparison of transfection efficiency and also actin provides loading information. Western blots were performed with anti -p180 (N-19), -MDM2 (Ab1, clone IF2), -p48 (PRIM1), -p58 (PRIM2) antibodies. Cells were treated either with DMSO or MG132 4 hours prior to harvesting as indicated.

3.5 *In vivo* NEDDylation assay to examine MDM2 involvement in mediating p180 modification

MDM2 has been shown to mediate ubiquitylation of itself and of many of its interacting proteins (64,151). Recently, several reports have linked MDM2 with NEDDylation, a process during which NEDD8 protein (a ubiquitin-like protein) is covalently linked to its substrates. Apart from promoting auto-NEDDylation, MDM2 has been reported to promote NEDDylation of p53 and of full-length (TA) p73, resulting in abrogation of their transcriptional activities (135, 136).

Evidence presented in this thesis suggests that MDM2 can bind to p180 and forms *in vivo* complexes with p180, therefore one possible consequence of this might be MDM2 mediated NEDDylation of p180. In order to test this hypothesis, we utilized a 6xHis-tagged NEDD8 construct (kindly provided by Dr. D. Xirodimas) for *in vivo* NEDDylation assays performed essentially as previously described for *in vivo* ubiquitylation assays in section 2.14.

H1299 cells were transfected with combinations of 6xHis-tagged NEDD8, MDM2 and p180 expression vectors. We wanted to examine whether p180 would be affinity purified with 6xHis-tagged NEDD8 by Ni-NTA, in either the presence or absence of transiently transfected MDM2 (Figure 3.5.1 lanes 3 and 4 respectively). By using an MDM2 RING finger mutant we were anticipating overcoming potential endogenous effect of wild type MDM2 on promoting p180 NEDDylation following the same argument we used previously for *in vivo* ubiquitylation (Figure 3.5.1, lane 5). Lanes 1, 2, 6 and 7 presented in Figure 3.5.1 serve as controls for the assay, since 6xHis-tagged NEDD8 was not transfected and thus only proteins that bind non-specifically to Ni-NTA agarose should be detected here. Finally, lane 4 serves as a

positive control (Figure 3.5.1) since, MDM2 was transfected along with 6xHis-tagged NEDD8 (and p180) and was expected to be present as a substrate for NEDD8 in lane 4 of Ni-NTA purified panel (Figure 3.4.3). This would confirm assay specificity, since MDM2 was previously shown to mediate its own NEDDylation (135).

Transfected cells were processed as described in Materials and methods (section 2.14), and harvested after 48 hours and then divided into two aliquots: one, aliquot consisting of 10% of harvested cells was used for western blot analysis, a second aliquot consisting of 90% of the sample was processed for the *in vivo* NEDDylation assay. In order to purify proteins conjugated to 6xHis-tagged NEDD8, lysates from 90% aliquots were incubated with Ni-NTA beads for 4 hours, washed and eluted with sample buffer supplemented with 200mM imidazole. Eluted samples along with cells lysates were subjected to SDS-PAGE followed by western blot analysis with antibodies against p180 (N-19) and MDM2 (Ab1, clone IF2) (Figure 3.5.1).

In Figure 3.5.1, MDM2 auto-NEDDylation is clearly detectable (see lane 4, Ni-NTA purified panel) as is the inhibitory effect of expression of an MDM2 RING finger mutant upon MDM2 auto-NEDDylation. The weaker signal in lane 5 likely derives from continued NEDDylation of the mutant by endogenous wild type MDM2 protein (Ni-NTA purified panel). As previously presented for the in vivo ubiquitylation assay (see Figure 3.4.4), we used ECL™ Advance Western Blotting Detection System (GE Healthcare Life Sciences) to increase the sensitivity of detection of p180 which can then be detected in all lanes. Comparing lanes 2 and 3

suggests that p180 may be a substrate for NEDDylation by an endogenous enzyme. Comparing lanes 3 and 4 suggests that this enzyme is not MDM2.

In summary, results obtained for both *in vivo* ubiquitylation and NEDDylation assays do not provide clear evidence for these modifications of p180. Although a number of MDM2 interacting proteins are subjected to ubiquitylation and/or NEDDylation due to MDM2 activity, we cannot find clear evidence that this occurs for p180. Nevertheless, data shown in Figure 3.4.6 do suggest that some p180 may be ubiquitylated albeit that MDM2 seems to play no positive role in this process. One potential explanation for our somehow equivocal results in cells may be that DNA polymerase α exists in more than one form. Two differentially phosphorylated forms have been described and it is possible that MDM2 interacts preferentially with one of these. The studies that follow represent our investigation of this question.

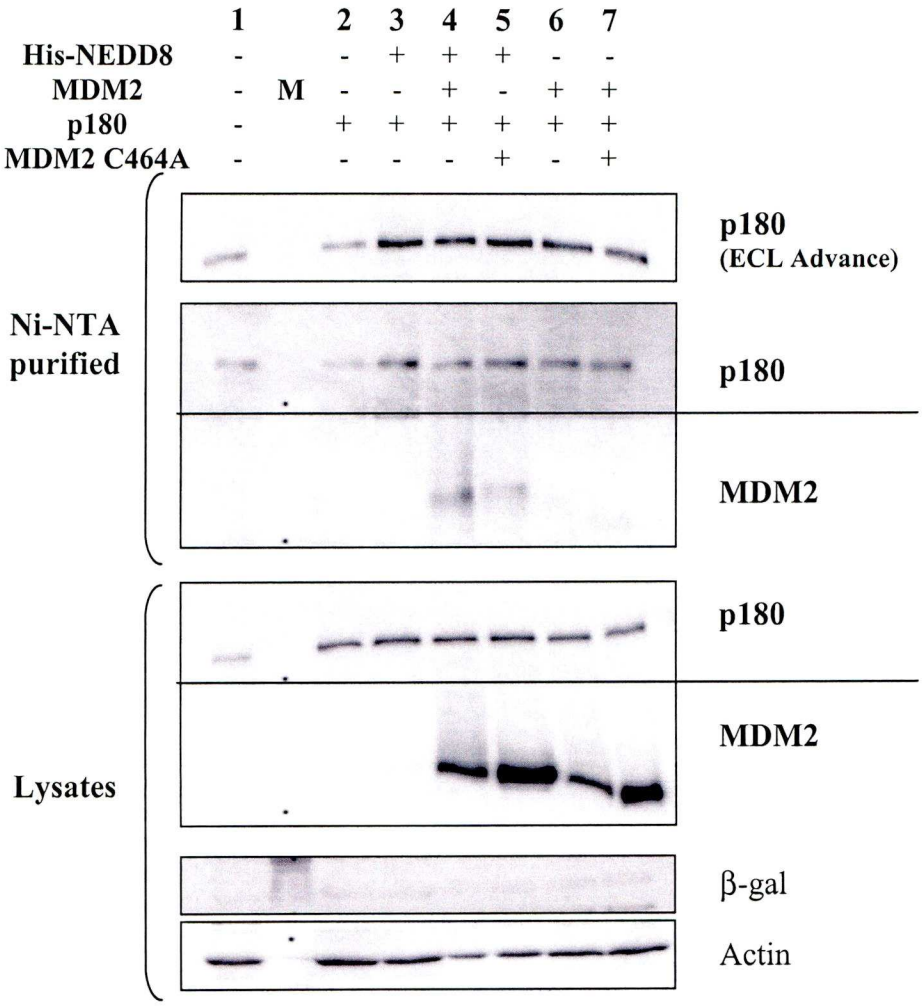


Figure 3.5.1 *In vivo* NEDDylation assay with 6xHis-tagged NEDD8, p180 and MDM2. H1299 were tranfected with 6xHis-tagged NEDD8 (5 μg), p180 (10 μg) and MDM2 (10 μg) using GeneJuice™ reagent in several combinations, as indicated. Ni-NTA purified section presents proteins purified with 6xHis-tagged NEDD8 on Ni-NTA agarose beads; Lysates section show effect of cell's transfection, with β-galactosidase (2μg) as a transfection efficiency control and actin as an loading control. Western blot was performed with anti -p180 (N-19), -MDM2 (Ab1, clone IF2), -actin (C-2) and -β-galactosidase (Ab1, clone 200-193) antibodies. ECL Advance Western Blotting Detection System was used to provide greater sensitivity (GE Healthcare) as indicated.

3.6 MDM2 interacts preferentially with hypophsophorylated DNA polymerase α

Among many reports published on DNA polymerase α, in particular one by Dehde S. *et al.* (116) has drawn our attention. This report described studies performed by the group of Professor Irene Dornreiter, which revealed that DNA polymerase α exists in two subpopulations defined by its phosporylation status. Furthermore, these two subpopulations of DNA polymerase α display different functions during replication, as proposed by Prof. Dornreiter's group, the non- or hypophosphorylated form synthesizes the primer for leading strand, whereas the phosphorylated form is involved in lagging strand primer synthesis. It is noteworthy that both subpopulations of DNA polymerase α may be distinguished using different antibodies, as reported by Dehde S. *et al.* (116) - HP180-12 antibody recognizes non- or hypophosphorylated form, while phosphorylated form could be detected with SJK132-20 antibody.

Both antibodies were generously provided to us by Prof. Dornreiter and we have used them in immunoprecipitation experiments to determine whether there are any differences in interaction between MDM2 and differentially phosphorylated forms of DNA polymerase α.

We initially performed immunoprecipitation on untransfected Clone 9 cells, which are derived from H1299 cells but express high levels of MDM2 (cells were stably transfected with pCMVNeoBamMdm2) and do not express p53. The immunoprecipitation procedure was carried out as described in Materials and methods (section 2.10) with HP180-12 (recognizes hypophosphorylated DNA polymerase α) and SJK132-20 (recognizes phosphorylated DNA polymerase α)

antibodies used along with isotype control (CD20). Samples were subjected to SDS-PAGE analysis followed by western blot with appropriate antibodies as presented in Figure 3.6.1.

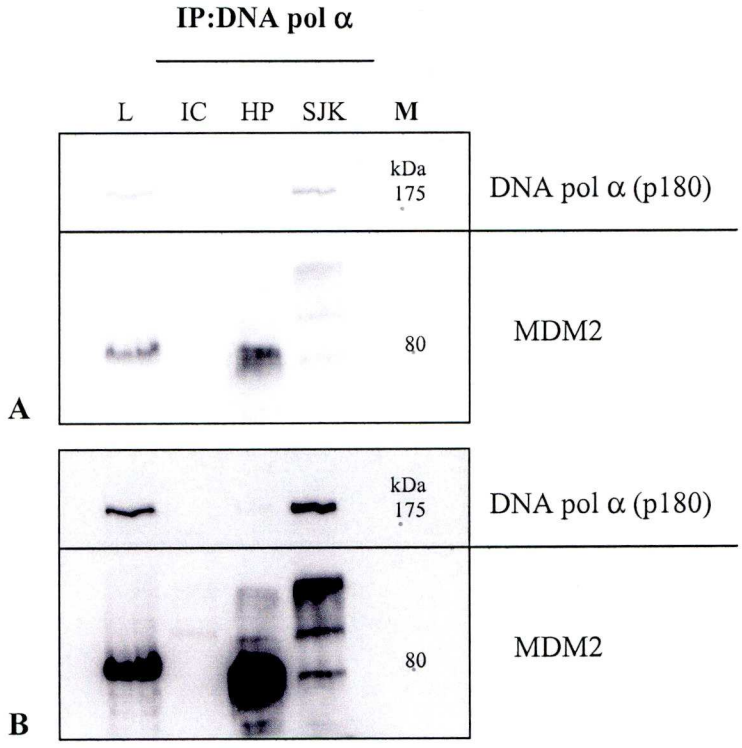


Figure 3.6.1 MDM2 interacts preferentially with hypophosphorylated DNA polymerase α in Clone 9 cells. Western blot of immunoprecipitation experiment performed on 2.5 mg of total protein from untransfected Clone 9 (H1299) cells with HP180-12 antibody and SJK132-20 recognizing non/hypophosphorylated or phosphorylated DNA polymerase α, respectively. Western blot performed on samples from lysates (L), isotype control (IC) – CD20 (Leu-16), HP180-12 (HP) and SJK132-20 (SJK) with anti-p180 (N-19) and anti-MDM2 (Ab1, clone IF2) antibodies (**A** - short exposure; **B** - long exposure); M – indicates protein molecular weight marker.

In Figure 3.6.1 we can see that the main MDM2 band (c.90kDa) is clearly present in the sample immunoprecipitated with HP180-12 (Figure 3.6.1), whilst there

is little of this form of MDM2 present in the sample immunoprecipitated with SJK132-20. It is noteworthy that more p180 is precipitated with the phospho-specific antibody than with the hypophosphorylated specific antibody and this makes the already apparent differences between MDM2 precipitations even more striking. In addition, the band pattern for MDM2 protein precipitated with SJK132-20 antibody as presented in the Figure 3.6.1 appeared to be unusual. In summary, it appears likely that MDM2 preferentially binds to the non- or hypophosphorylated form of DNA polymerase α in Clone 9 cells in culture.

To investigate this possibility further, we added two additional cell lines to this analysis. Human embryonic kidney cells 293 (HEK293) cells express high levels of MDM2 which makes the detection of co-precipitating proteins easier and we also added H1299 cells which express relatively low levels of MDM2 (especially in comparison to Clone 9 cells). The immunoprecipitation experiment was performed on these three cell lines (H1299, Clone 9 and HEK293) as described in Materials and methods (section 2.10) with HP180-12 and SJK132-20 antibodies used along with isotype control (CD20). Samples were subjected to SDS-PAGE analysis followed by western blot with appropriate antibodies as presented in Figure 3.6.2.

Results presented in Figure 3.6.2 confirmed the previously made observation that MDM2 interacts preferentially with non- or hypophosphorylated DNA polymerase α, since on the presented blots, there was abundant MDM2 protein band in the lane corresponding to samples immunoprecipitated with HP180-12 antibody, for each cell line tested (Figure 3.6.2 A,B,D). Again, an unusual MDM2 band pattern was displayed in lanes corresponding to SJK132-20 samples, and use of a second anti–MDM2 antibody (Ab6) for western blot analysis (Figure 3.6.2D) confirmed the

identity of these as unusual MDM2 bands (Figure 3.6.2A,B). We also observed as we had previously that the SJK132-20 (phosphorylation specific) antibody precipitated more p180 than the HP180-12 antibody thus the observed bias towards MDM2-hypophosphorylated p180 is likely to be greater than it at first appears.

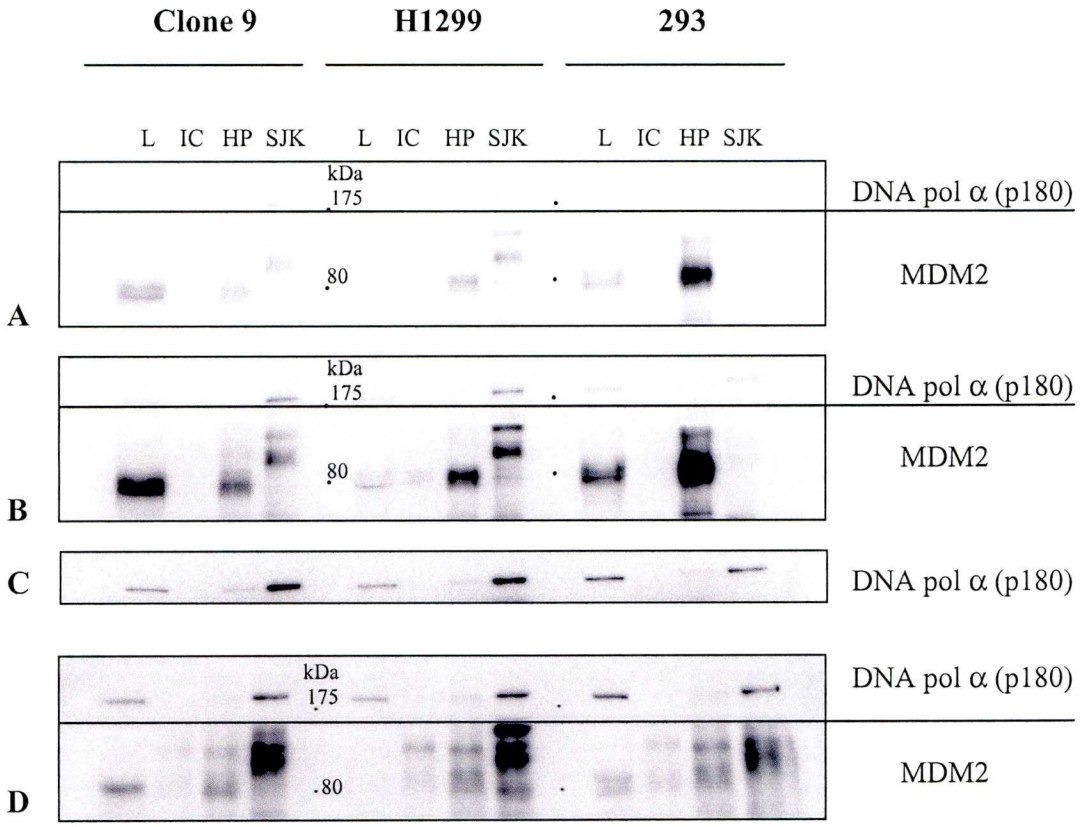


Figure 3.6.2 MDM2 interacts preferentially with hypophosphorylated DNA polymerase α in Clone 9, H1299 and HEK293 cells. Western blot of immunoprecipitation experiment performed on 2.5 mg of total protein from untransfected Clone 9, H1299 and 293 cells with HP180-12 antibody and SJK132-20 recognizing non/hypophosphorylated or phosphorylated DNA polymerase α, respectively. Western blot performed on samples from lysates (L), isotype control (IC) – CD20 (Leu-16), HP180-12 (HP) and SJK132-20 (SJK) with anti-p180 (N-19) and anti-MDM2 (Ab1, clone IF2) antibodies (**A** - short exposure; **B** - long exposure); **C** - anti-p180 (N-19) – very long exposure; **D** - anti-p180 (N-19) and anti-MDM2 (Ab6, clone 5B10C) antibodies.

In summary, we conclude that formation of a complex between MDM2 and non- or hypophosphorylated subpopulation of DNA polymerase α in the cells in culture seems to occur independently of p53, the expression level of MDM2 and is not limited to cells of one type of origin.

DNA polymerase α has previously been shown to interact with SV40 T large antigen (45, 47, 48) we wondered therefore whether the presence of T antigen may affect the interaction between MDM2 and DNA polymerase α. HEK293T cells stably express SV40 large T antigen and were used for an immunoprecipitation experiment along with HEK293 cells. The immunoprecipitation experiment was performed on untransfected HEK293 and HEK293T cells as described in Materials and methods (section 2.10) with HP180-12 and SJK132-20 antibodies used along with isotype control (CD20). Samples were subjected to SDS-PAGE analysis followed by western blot with appropriate antibodies as presented in Figure 3.6.3.

As Figure 3.6.3 shows, abundant MDM2 protein band can be detected in both cell lines following immunoprecipitation with HP180-12 antibody. Thus it appears that MDM2 interacts preferentially with non/hypophosphorylated subpopulation of DNA polymerase α in both cell lines. In addition, it seems that the presence of T antigen, does not affect formation of a complex between MDM2 and the non- or hypophosphorylated form of DNA polymerase α (Figure 3.6.3A).

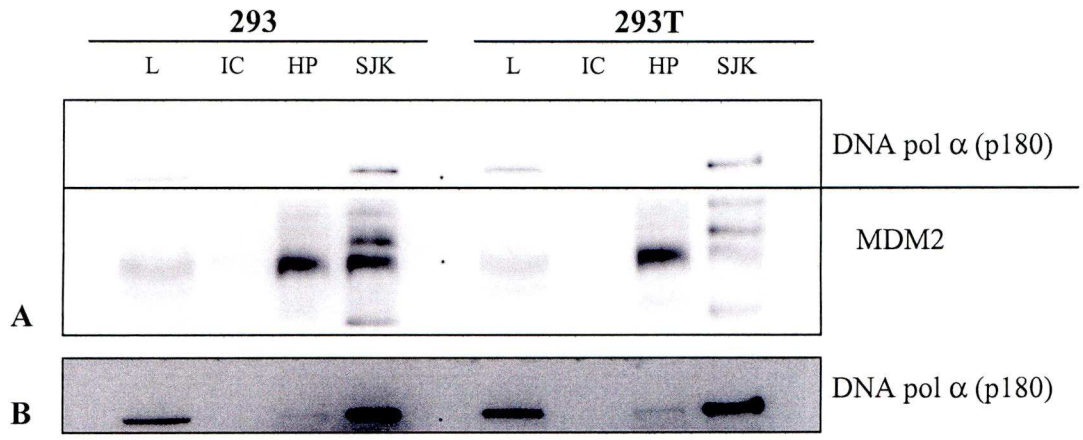


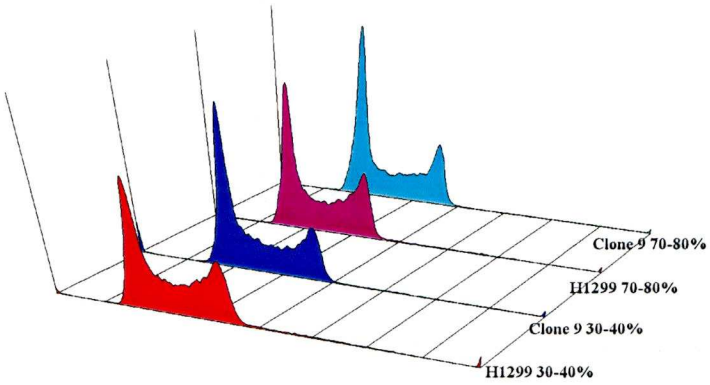
Figure 3.6.3 MDM2 interacts preferentially with hypophosphorylated DNA polymerase α in HEK293 and HEK293T cells. Western blot of immunoprecipitation experiment performed on 2.5 mg of total protein from untransfected 293 and 293T cells with HP180-12 antibody and SJK132-20 recognizing non/hypophosphorylated or phosphorylated DNA polymerase α, respectively. Western blot performed on samples from lysates (L), isotype control (IC) – CD20 (Leu-16), HP180-12 (HP) and SJK132-20 (SJK) with anti-p180 (N-19) and anti-MDM2 (Ab1, clone IF2) antibodies (**A** - short exposure); **B** with anti-p180 (N-19) - long exposure.

Our observations that MDM2 interacts preferentially with one of DNA polymerase α subpopulations may have implications for processes regulated by DNA polymerase α phosphorylation i.e. initiation of DNA replication. As stated previously, the hypophosphorylated form of DNA polymerase α is linked with synthesizing the primer for the leading strand during initiation of replication (116). The fact that non-/hypophosphorylated subpopulation of DNA polymerase α is involved in forming a complex with MDM2 in the cell may suggest a role of MDM2 in the replication initiation process via interaction with that DNA polymerase α subpopulation. Clearly, further research needs to be done in order to shed light on this subject.

3.7 Cell cycle profile analysis on cells expressing different amounts of MDM2

Research performed on transgenic mice has provided information that overexpression of MDM2 may trigger several rounds of replication without cell division, leading to polyploidy (97). These findings indicate an involvement of MDM2 in the processes of DNA synthesis and/or cell division, which is independent of p53 and E2F1 (97, 139). The new observations presented in this thesis, regarding MDM2 forming a complex with hypophosphorylated DNA polymerase α, may provide a part of the mechanism for this effect of MDM2.

In the first instance, to investigate whether MDM2 alters DNA synthesis/initiation of DNA synthesis we analysed the cell cycle profile of cells expressing differing levels of MDM2. Whilst this cannot determine the rate or initiation rate of DNA synthesis, it might provide evidence that MDM2 has an impact on the cell cycle (clearly this need to be done in p53 null cells to exclude the well known effects of p53 on the cell cycle). We were interested in elucidating whether different levels of MDM2 expressed in the same type of cells may influence their cell cycle profiles. In order to address this question flow cytometry analysis was performed on H1299 and Clone 9 (H1299) cells expressing different amounts of MDM2. Cells were harvested by trypsinization at 30 – 40 and 70 – 80% confluency and processed as described in Materials and methods (section 2.15), followed by samples' analysis in a Beckman Coulter Epics XL flow cytometer using FL2 (propidium iodide) channel.



A

Cells /%	G1	S	G2
H1299 (30-40%)	33.6	55.6	10.8
Clone 9 (30-40%)	38.7	50.2	11.1
H1299 (70-80%)	35.8	52.6	11.6
Clone 9 (70-80%)	40.2	49.2	10.6

B

Figure 3.7.1 Flow cytometric analysis of H1299 and Clone 9 cells. Cells were harvested at 30-40 and 70-80% confluency, stained with propidium iodide. Twenty thousand events were collected per condition. Data were analyzed using **A**) WIN MDI software and **B**) Cylchred software.

Figure 3.7.1 presents results from flow cytometric analysis performed on untreated/untransfected H1299 and Clone 9 (H1299) cells. Based on the chart and values shown in the table there do not appear to be major differences in the cell cycle profiles of these cells.

In the next step we wanted to examine whether cell cycle profiles could be affected by reducing endogenous levels of MDM2. Hence, *MDM2* small interfering RNA (siRNA) #1 was delivered to H1299 and Clone 9 (H1299) cells, while

scrambled siRNA (base composition matched to *MDM2* siRNA #1) served as a negative control for MDM2 knockdown. The siRNA transfection was performed as described in Materials and methods (section 2.5), then cells were subjected to SDS-PAGE followed by western blot analysis, in order to verify effectiveness of the procedure. As shown in Figure 3.7.2 reducing the endogenous MDM2 expression level with *MDM2* siRNA #1 was successful, both in H1299 and Clone 9 (H1299) cells.

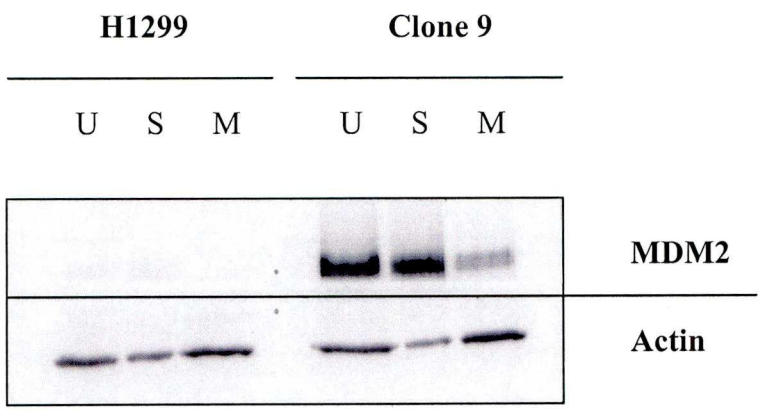


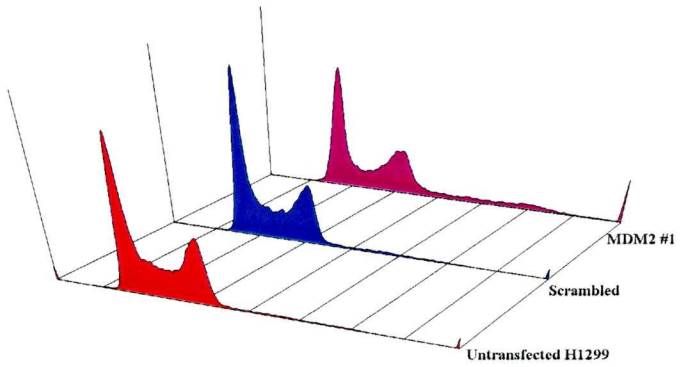
Figure 3.7.2 MDM2 knock-down with siRNA in H1299 and Clone 9 cells. Western blot analysis of siRNA transfected H1299 and H1299-Clone 9 cells performed with anti-MDM2 (Ab1, clone IF2) and anti-actin (C-2) antibodies; lane marked with U represents untransfected cells; S – cells transfected with scrambled siRNA and M - cells transfected with MDM2 #1 siRNA, actin served as a loading control.

Having tested the efficacy of siRNA knock-down of MDM2 in H1299 and Clone 9 (H1299) (Figure 3.7.2), remaining cells from the same transfection were analysed in a Beckman Coulter Epics XL flow cytometer using FL2 (propidium iodide) channel.

A considerable difference in cell cycle profiles was observed both for H1299 and Clone 9 (H1299) cells with reduced endogenous levels of MDM2 (transfected

with *MDM2* siRNA #1). It seemed that number of cells in S phase was reduced by approximately 10% when compared to either untransfected cells or to cells transfected with scrambled siRNA (Figure 3.7.3).

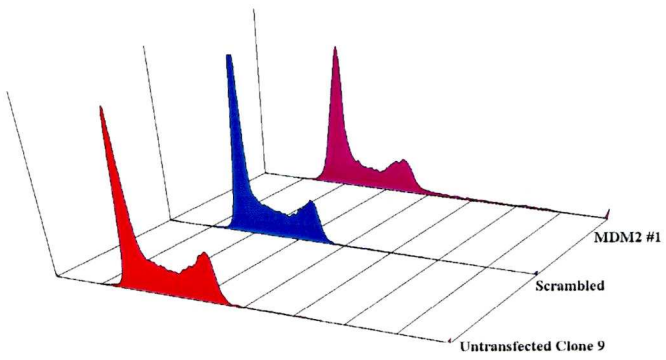
The results shown in Figure 3.7.3 appear to indicate a role for MDM2 in regulating the cell cycle. Given the function of DNA polymerase α in initiation of DNA synthesis, we proposed to examine the incorporation of BrdU as a means of measuring the rate of DNA synthesis, however since time was running out for this project and this technique was not already established in our laboratory, we thought it might be worthwhile to measure the total DNA synthesis in a population of cells using a simpler assay. We therefore measured [^{3}H]-thymidine incorporation, an assay usually used to measure cell proliferation rate based on incorporation of [^{3}H]-thymidine into nucleic acid.



A

H1299	G1	S	G2
Untransfected	28.1	55.2	16.7
Scrambled	30	51.6	18.4
MDM2 #1	35.2	41	23.8

B



C

Clone 9	G1	S	G2
Untransfected	36.1	53.2	10.7
Scrambled	39.5	47.5	13
MDM2 #1	42.8	38.3	18.9

D

Figure 3.7.3 Flow cytometric analysis of H1299 and Clone 9 cells with knocked-down MDM2. A, B) Flow cytometric analysis of H1299; **C, D)** Clone 9 (H1299) cells transfected with scrambled and MDM2 #1 siRNA, stained with propidium iodide. Twenty thousand events were collected per condition. Data were analyzed using **A, C**) WIN MDI software and **B,D**) Cylchred software.

H1299 cells were transfected with either *MDM2* siRNA #1 or scrambled siRNA (matched to *MDM2* siRNA #1). In addition, cells were treated with siRNA transfecting agent - Lipofectamine in order to detect any effect of this reagent on cell proliferation. The siRNA transfection was performed as described in Materials and methods (section 2.5), then cells were subjected to SDS-PAGE followed by western blot analysis, in order to verify effectiveness of performed procedure. As shown in Figure 3.7.4 *MDM2* siRNA #1 successfully reduced the level of MDM2 protein in H1299 cells.

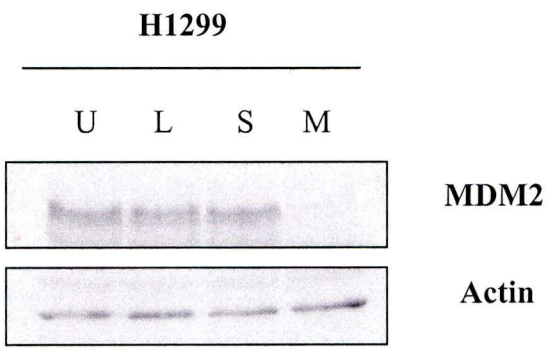
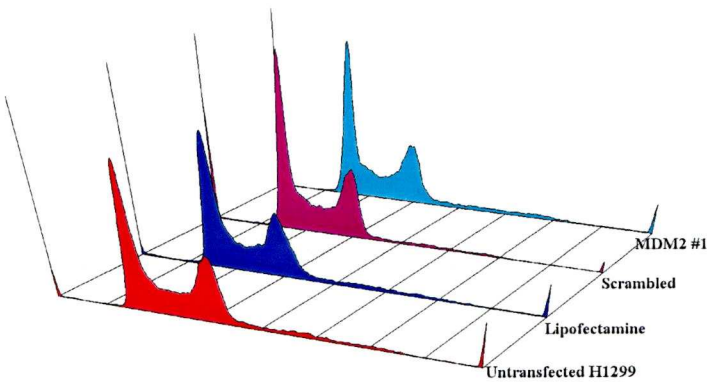


Figure 3.7.4 MDM2 knock-down with siRNA in H1299. Western blot analysis of siRNA transfected H1299 cells performed with anti-MDM2 (Ab1, clone IF2) and anti-actin (C-2) antibodies; lane marked with U represents untransfected cells; L – Lipofectamine treated cells; S – cells transfected with scrambled siRNA and M - cells transfected with MDM2 #1 siRNA, actin served as a loading control.

Cells from the same transfection were also processed for [3H]-thymidine incorporation as described in Materials and methods (section 2.15), followed by analysis in a Beckman Coulter Epics XL flow cytometer using FL2 (propidium iodide) channel.

Results presented in Figure 3.7.5 do not fully support previously made observations (Figure 3.7.3). As previously there is a lower percentage of S and a high percentage of G2 cells in the population treated with *MDM2* siRNA#1, but on this occasion the effect is reduced and it is not possible to determine the significance of this effect.



A

H1299	G1	S	G2
Untransfected	33.4	48.7	17.9
Lipofectamine	34.1	47.7	18.2
Scrambled	33	50.1	16.9
MDM2 #1	33.9	44.7	21.4

B

Figure 3.7.5 Flow cytometric analysis of H1299 cells with knocked-down MDM2. Flow cytometric analysis of H1299 cells transfected with scrambled and MDM2 #1 siRNA, stained with propidium iodide. Twenty thousand events were collected per condition. Data were analyzed using: **A)** WIN MDI software and **B)** Cylchred software.

The [3H]-thymidine incorporation assay was performed alongside with flow cytometry analysis by incubating H1299 cells with [3H]-thymidine for 2 hours, followed by scintillation counting in order to measure [3H]-thymidine incorporation into nucleic acid. As shown in Figure 3.7.6, although the mean signal for *MDM2* siRNA #1 treated cells is lower, this is not significant, which is in agreement with the flow cytometry results presented in Figure 3.7.5.

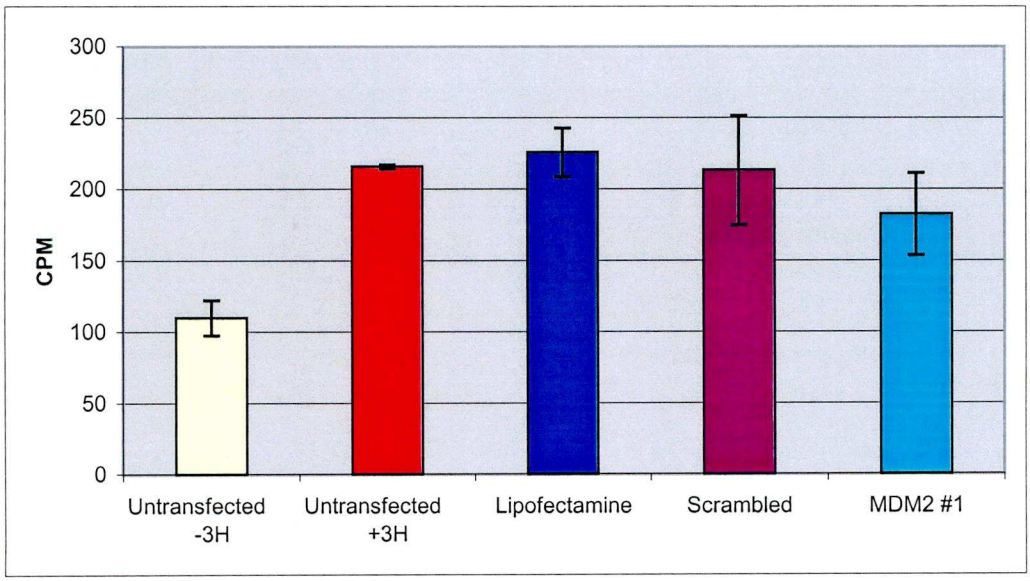


Figure 3.7.6 [3H] thymidine incorporation assay performed on H1299 cells. Coloured boxes indicate [3H] thymidine incorporation in H1299 cells: untransfected, treated with Lipofectamine, transfected with scrambled and MDM2 #1 siRNA (CPM – counts per minute). Data are shown as mean $\pm$ standard error of the mean.

In summary, the rather preliminary results presented in this section of the thesis do not succeed in addressing the question, whether knockdown of MDM2 may alter the cell cycle profiles as a consequence of altering DNA synthesis/initiation of DNA synthesis. However, in the light of our observations that MDM2 selectively forms a complex in a cell with the hypophosphorylated form of DNA polymerase α,

which is engaged in synthesizing first primer on the leading strand during DNA replication, and previous findings indicating a role for MDM2 in the regulation of DNA synthesis independent of p53 and E2F1 (97, 139), further research definitely needs to be done in order to elucidate the possible role of MDM2 – DNA polymerase α interaction in cells.

CHAPTER IV

DISCUSSION

The aim of work presented in this thesis was to investigate interaction between MDM2 and DNA polymerase α. These studies were prompted following the discovery that MDM2 co-purifies with DNA polymerase α made during course of studies in our research group on the interaction between MDM2 and DNA polymerase ε (58,59) (these two protein complexes co-purify in partially overlapping fractions from mammalian cells). Preliminary data that contributed to the planning of the work contained in this thesis project, consisted of experiments performed by former members of our research group – Dr. Maria Maguire and Dr. Paul Nield. They showed that DNA polymerase α, based on detection of its catalytic subunit (p180), co-purifies with 6xHis-tagged MDM2 from a nickel charged column by affinity chromatography (Figure 3.1.1). In addition Dr. Maguire showed that MDM2 could be detected in a complex with DNA polymerase α as demonstrated by the co-immunoprecipitation of these endogenous proteins from HEK293 cells (Figure 3.2.1). However, since these experiments do not provide information about direct interaction between MDM2 and DNA polymerase α, we wanted, in the first instance, to address this issue.

In order to examine the direct interaction between MDM2 and DNA polymerase α and to define protein regions involved in protein binding, we have utilized an *in vitro* binding assay, which has been used previously in our laboratory (56,58). In this assay, one partner protein is radiolabeled, which allows visualization of the signal, while the other one is 6xHis-tagged which aids purification. To

successfully purify 6xHis-tagged MDM2 and MDM2 mutants, it was necessary to first determine optimal conditions for expression and purification of these proteins. Based on results shown in Figures 3.1.2-5, we decided that the optimal induction time with IPTG for expression of 6xHis-tagged MDM2 was 4 hours, which conferred high protein expression levels, and in addition may result in less toxicity and protein degradation than might arise at later time points. For the purification of MDM2 proteins adjustments were made to the wash buffers such that the pH was raised for MDM2 fragments since they appeared to display reduced affinity for Ni-NTA beads (Figure 3.1.3). This approach works because by increasing the pH of the buffers we reduced protonation of histidine residues in the 6xHis tag and thus this retains the electrostatic attraction between histidine and nickel ions.

Once proteins were successfully purified (Figures 3.1.4) *in vitro* binding assays were performed with protein partners as presented in Table 3.1.1. Since the amounts of purified 6xHis-tagged MDM2 proteins were variable (Figure 3.1.4), in particular carboxy-terminal half of MDM2 being present on the Ni-NTA beads at higher levels than the other proteins, we attempted to normalize these amounts as presented in Figure 3.1.9. Nevertheless for the assay we decided to use both – greater amount of carboxy-terminal MDM2 (Figure 3.1.4) and normalized amount of carboxy-terminal MDM2 (Figure 3.1.9) along with other 6xHis-tagged proteins. Results presented in Figure 3.1.10 suggest that full length p180, as well as amino- and carboxy-terminal halves of p180 preferentially bind to amino-terminal half of MDM2, consisting of the first 246 amino acids, as well as to full length MDM2. These results may not be surprising, since the amino-terminal region of MDM2 is known to be involved in MDM2 binding to a number of proteins, including DNA

polymerase ε (58), p53 and E2F1 (151). In addition, despite the fact that we have also used an excess of the carboxy-terminal half of MDM2 compared to full length or amino-terminal half of MDM2 (Figure 3.1.4), and yet did not observe interaction between p180 and the carboxy-terminal half of MDM2, strengthens the evidence for preferential p180 binding to the amino-terminus of MDM2.

To further analyse the role of the MDM2 amino-terminus in binding to p180, an MDM2 mutant lacking the amino-terminal 166 amino acids (Δ166 MDM2) of MDM2 was examined in the *in vitro* binding assay (Figure 3.1.11). Similarly, to what we have observed previously with carboxy-terminal half of MDM2 (Figure 3.1.4), Δ166 MDM2 protein was present on the beads at a much higher level than the other 6xHis-tagged proteins examined for comparison (Figure 3.1.11A). Hence, we normalized the amount of proteins as presented in Figure 3.1.11B, but as before we have used both the high level and a reduced normalized amount of Δ166 MDM2 protein, along with other 6xHis-tagged proteins for the assay (3.1.11). Results presented in Figure 3.1.12 show that there is no detectable interaction between Δ166 MDM2 and p180. These results suggest that the amino terminal 166 aa of MDM2 are necessary for binding to the catalytic subunit of DNA polymerase α (p180). This notion is further supported by the fact that Δ166 MDM2 protein was also present at higher levels than the other 6xHis-tagged protein in the assay, and yet interactions between Δ166 MDM2 and p180 were still not observed.

Based on the same approach, we attempted to determine the region of p180 that is necessary for interaction with MDM2. However, it seems that both amino- and carboxy-terminal halves of p180 bind equally well to the amino terminal region of MDM2 (Figures 3.1.10, 3.1.12), suggesting that multiple regions of p180 might be

involved in MDM2 binding. Multiple binding sites between MDM2 and an interacting protein are not unusual. For example, both ARF and p53 proteins display more than one site of interaction with MDM2 (200, 201).

After performing a set of *in vitro* studies, based on which we concluded that p180 subunit binds preferentially to amino-terminal part of MDM2 and that the amino-terminal 166 amino acids of MDM2 seems to be indispensable for this interaction, we wanted to further analyse this interaction in cells in culture by immunoprecipitation (IP). The IP method is based on antibody binding to a specific target protein which is precipitated using beads (in this instance agarose) coupled to an antibody capturing protein, in this case Protein G. Interacting proteins are then captured together with the primary protein and the complexes can then be interrogated by a number of means, the simplest being with a second antibody to the suspected binding partner. This method is relatively simple and can be sensitive, but it cannot be used to discriminate between direct and indirect protein interaction. We were not concerned with this issue in the present set of experiments since we had already determined the region/s of MDM2 and DNA polymerase α required for direct interaction through our *in vitro* studies (Figure 3.1.10, 3.1.12). It is also noteworthy, that formation of the complex between endogenous MDM2 and DNA polymerase α in the cells in culture has been already demonstrated by the immunoprecipitation experiment performed by Dr. Maria Maguire (Figure 3.2.1).

To further examine MDM2 and p180 interaction, particularly to determine the region of MDM2 necessary for this interaction *in vivo*, a series of HA-tagged MDM2 carboxy-terminal deletion mutants (generated by Dr. Tim Devling) were used. These mutants contain amino-terminal 335, 280, 230, and 166 amino acids of MDM2

protein. Of particular interest was the MDM2 mutant containing only the first 166 aa, since this region was required for binding to p180 *in vitro*. Prior to the immunoprecipitation assay, p180 and MDM2 carboxy-terminal deletion mutants were transfected into H1299 cells, which were chosen because they are easily transfectable and also do not express p53. Since p53 was shown to interact with p180 (13,14) as well as MDM2 (5), we wanted to exclude any potential involvement of p53 in p180-MDM2 interaction. Immunoprecipitation was performed with an anti-p180 antibody and co-immunoprecipitated complexes analysed by western blotting. Results of these experiments (Figure 3.2.10A) show that p180 co-immunoprecipitates with all of the MDM2 carboxy-terminal deletion mutants described, supporting conclusions from *in vitro* studies suggesting that amino-terminal 166 amino acids fragment of MDM2 is required for binding to p180. These studies also demonstrated that p180 and carboxy-terminal MDM2 mutants form a complex in cells in p53-independent manner, as expected from the *in vitro* binding experiments.

In order to refine the MDM2 region involved in interaction with p180, an MDM2 mutant lacking amino-terminal 49 amino acids (Δ49MDM2) was used in an immunoprecipitation experiment. This IP experiment demonstrated that p180 was co-immunoprecipitated with Δ49MDM2 from H1299 cells, suggesting that the region of MDM2 between aa 50 and 166 seems to be sufficient for binding to p180 (Figure 3.2.10A). Finally, we created and tested an MDM2 mutant lacking the amino-terminal 166 amino acids (Δ166MDM2) for immunoprecipitation experiments with p180. Surprisingly and contrary to our expectations, p180 was co-immunoprecipitated with Δ166MDM2 as shown in Figure 3.1.10B. It is difficult to

interpret this result, however, it is possible that another protein (or proteins) mediate MDM2-p180 interaction or that MDM2 may not only interact with p180 but also with other subunits of DNA polymerase α. We wanted to further analyse the latter possibility. As we had demonstrated MDM2 appears to interact directly with the catalytic p180 subunit of DNA polymerase α *in vitro*, and additionally, we could also detect a complex containing the p180 subunit in cells. However, since the MDM2 mutant lacking the first 166 amino acids was also shown to form a complex with p180, but does not interact directly with this subunit *in vitro*, we concluded that this latter complex probably interacts with other proteins and the most likely candidates for this would be other subunits of DNA polymerase α. Unfortunately, our attempts to test antibodies for the other subunits of DNA polymerase α, only succeeded in demonstrating that some of those tested detected multiple bands on a western blot and moreover many of these were not of the expected molecular weight (data not shown) and some did not detect any protein (Figure 3.4.6) .

Since we had established that MDM2 interacts with the catalytic subunit p180 which harbours the DNA polymerase activity, we wanted to investigate whether MDM2 had any affect on the enzymatic activity of p180 by utilizing a DNA polymerase assay. The assay enables us to measure enzyme activity based on incorporation of [3H]dTTPs. In earlier studies it had been demonstrated that MDM2 can stimulate the enzymatic activity of the DNA polymerase α-related DNA polymerase ε (59) by 20-40-fold and therefore it was not unreasonable to suspect that MDM2 might stimulate DNA polymerase α as well.

DNA polymerase activity assay was performed with catalytic subunit of DNA polymerase α (p180) and MDM2 protein which were expressed in insect cells using

a baculovirus system with the proteins subsequently purified by virtue of their 6xHis tags using Ni-NTA beads. Initial experiments suggested that the DNA polymerase activity of the p180 subunit was stimulated upon addition of MDM2 enriched protein fractions as shown in Figure 3.3.4. However, unlike previous studies with DNA polymerase ε, these latter studies were based for purely practical reasons on less highly purified MDM2 and also the stimulatory effect was only moderate, being at best a few-fold. The possibility exists, that during MDM2 purification process other contaminant proteins were eluted together with MDM2. Therefore, the stimulatory effect observed in Figure 3.3.4 might be an effect of protein or proteins other than MDM2, which could either possess intrinsic polymerase activity or mediate the stimulatory effect by acting upon p180. Since our main concern here was the purity of MDM2 used for the assay (Figure 3.3.7), we decided to include specificity protein controls consisting of proteins purified from uninfected insect cells under exactly the same conditions as MDM2 used for the assay (Figures 3.3.5, 3.3.7). We also examined whether proteins from uninfected insect cells or of MDM2 fractions possess intrinsic polymerase activity, but we have not detected any evidence of such activity, except of course in fractions from insect cells expressing p180 fraction (Figure 3.3.8). When p180 activity was assayed with proteins purified from the uninfected insect fraction, adjusted to have comparable amounts of background protein as MDM2 fraction, we observed that the catalytic activity of p180 was stimulated to the same extent as with MDM2 (Figure 3.3.10). Based on these results, it was not clear whether MDM2 is responsible for the stimulatory effect on p180 activity or whether it is mediated by other protein/proteins. To try to examine this question further we tested antibodies against MDM2 that could potentially prevent

MDM2 from stimulating p180. We envisaged that this might work either through competitive inhibition of MDM2-p180 interaction or by having structural effects on MDM2 that might for example prevent potential allosteric changes in MDM2 that could be required for the stimulatory effect. Antibodies were added to purified MDM2 and p180 in a DNA polymerase assay. Results of these experiments show that stimulatory effect on p180 enzymatic activity was not diminished upon addition of MDM2 antibodies. Unfortunately this result is ambiguous suggesting that either the antibodies used had no effect on MDM2 stimulation or that stimulation is mediated by other proteins purified from the insect cells rather than MDM2. It is worth noting that in Figure 3.3.17 this approach, that is, use of an antibody to inhibit the polymerase activity of p180 was used successfully. Finally, we decided to test whether MDM2 purified from bacterial cells could alter the activity of p180. The rationale for this approach was as follows: we have recently performed experiments using GST-MDM2 and these demonstrated (as others have described) that MDM2 can retain its E3 ligase activity for approximately 24h, as verified by *in vitro* ubiquitylation assays (134). Thus we could test whether an active and an inactive form (stored at -80°C) of bacterially expressed GST-MDM2 would have any impact on p180 activity. In addition to this, purification of MDM2 from a different system based upon an organism other than the insect cell system would provide not only purer MDM2 but also a protein preparation with a different set of contaminants. Moreover, the stimulatory effect of bacterially purified MDM2 was also observed previously on DNA polymerase ε (59). In the assay p180 was mixed either with enzymatically inactive or active preparations of MDM2. The results demonstrated no effect on the activity of p180 with either form of MDM2 (Figures 3.3.13, 3.3.16). In

contrary to previous findings on the effect of MDM2 on DNA polymerase ε (59) we failed to obtain clear evidence for any effect of MDM2 on DNA polymerase α activity. A major problem we experienced was that the purity of MDM2 obtained from the insect cells fraction was demonstrably lower than that used previously in studies of DNA polymerase ε (59). Therefore, we collaborated with Prof. Stuart Linn again at U.C. Berkeley to examine the effect of this more highly purified MDM2 on DNA polymerase α. Based on the experiment performed by Prof. Linn it can be observed that the activity of DNA polymerase α was impressively stimulated upon addition of baculovirus expressed purified MDM2 as presented in Figure 3.3.17. These results suggest that MDM2 may indeed have a stimulatory effect on DNA polymerase α activity. This experiment, which clearly requires further examination was based upon purified DNA polymerase α holoenzyme obtained from large scale biochemical purification of DNA polymerase from HeLa cells (5 columns and c. 40 liters of cells). The magnitude of this stimulation may therefore derive from the more highly purified MDM2 used, or from the use of the holoenzyme, or both. Future studies will be aimed at further examining this.

After having tested the effect of MDM2 on DNA polymerase α activity, we wanted to determine whether MDM2 may affect the enzyme in other ways. As described in the Introduction chapter of this thesis, MDM2 possesses E3 ubiquitin ligase activity and mediates ubiquitylation and thus promotes proteasome-mediated degradation of several of its interacting partners. Therefore, we wanted to investigate whether MDM2 can mediate ubiquitylation of p180. Initially, it was important to determine whether p180 is subject to degradation via ubiquitin/proteasome-dependent pathway at all. Based on our findings with MCF-7, Clone 9 and H1299 cells treated with

MG132, an inhibitor of 26S proteasomes, it is possible that p180 might be stabilized in MCF-7 and Clone 9 cells, however not in H1299 (Figure 3.4.1-2). This may suggest a role for MDM2 in that process, since H1299 cells express relatively low levels of MDM2 especially comparing to Clone 9 cells. Overall though, the data do not provide clear evidence for degradation of p180 via the ubiquitin/proteasome-dependent pathway, particularly in the light of results from others which showed no evidence for p180 intracellular degradation (109). However, since MDM2 was also shown to promote mono-ubiquitylation of some of its interacting proteins (151), which does not result in proteasomal degradation, we wanted to examine the role of MDM2 in mediating that modification of p180. Noteworthy is the fact that mono-ubiquitylation mediated by MDM2 was shown to alter the enzymatic activity of DHFR (134), which suggests that similar effects might be detectable for p180 if it were to be modified by MDM2. To examine the question of whether p180 is ubiquitylated by MDM2 we performed a series of *in vivo* ubiquitylation assays. Based on results presented in Figures 3.4.3 and 3.4.4 there was a hint that p180 might be ubiquitylated either in presence or absence of MDM2. However these assays were significantly compromised by the non-specific binding of p180 to Ni-NTA beads and therefore we decided to use another approach to address this issue.

We also performed immunoprecipitation experiments to examine whether p180 was i) ubiquitylated and ii) whether this was MDM2 dependent. These experiments utilised HA-tagged ubiquitin, which was transfected along with p180 and MDM2 expressing constructs in various combinations. The results (Figure 3.4.5) did not detect any evidence that p180 is ubiquitylated. We therefore attempted to examine whether other subunits of DNA polymerase α complex might be subjected

to ubiquitylation.

We performed an *in vivo* ubiquitylation assay as we had previously, however on this occasion MG132 was also added to cells prior harvesting, in order to prevent protein degradation and hence increase the amount of ubiquitylated proteins in present in the cells. As shown in Figure 3.4.6 we failed to detect primase subunits of DNA polymerase α, probably due to the unsatisfactory quality of antibodies used for detection. However, more importantly we observed for the first time that p180 seems to be ubiquitylated in the absence of MDM2, whilst addition of MDM2 appears to inhibit p180 ubiquitylation (Figure 3.4.6). In addition, p180 non-specific binding to Ni-NTA beads was relatively lower than previously observed, probably due to improved signal to noise following from the use of MG132. MDM2 was previously shown to inhibit ubiqutylation of E2F1 and the mechanism of this appears to be that MDM2 binds to E2F1 and displaces SCF^{Skp2}, resulting in stabilization of E2F1 (155). Thus our results with DNA polymerase α and MDM2 in Figure 3.4.6 may represent a similar situation, but clearly further experiments will be required to investigate this.

Ubiquitylation is not the only modification mediated by MDM2 due to its E3 ligase activity. MDM2 is also capable of mediating NEDDylation, which is a process analogous to ubiquitylation, where a ubiquitin-like protein – NEDD8 is conjugated to its substrate. Thus far, apart from mediating its own NEDDylation, MDM2 has been observed to NEDDylate p53 and full-length (TA) p73, which resulted in inhibition of transcriptional activity of both targeted proteins (135,136). We wanted to examine whether MDM2 might mediate NEDDylation of p180 by performing *in vivo* NEDDylation assays with 6xHis-tagged NEDD8 (kindly provided by Dr. D. Xirodimas). As presented in Figure 3.5.1 the assay findings confirmed

auto-NEDDylation of MDM2, however it was impossible to determine whether p180 was NEDDylated as well, since the p180 background due to non-specific binding to Ni-NTA again compromised the assay with respect to p180.

Experiments performed to examine an effect of MDM2 on mediating p180 modifications provided us with ambiguous results. The p180 was observed to be ubiquitylated in cells, and rather than promoting this it appears as though MDM2 was engaged in inhibition of this process. One potential explanation for our seemingly equivocal results in cells may be that DNA polymerase α exists in more than one form. Therefore we focused our attention on findings regarding DNA polymerase α phosphorylation status and how this impacts upon its activity in DNA replication, as reported by Professor Irene Dornreiter's research group (116). It is noteworthy that DNA polymerase α is subject to modification by phosphorylation on its two biggest subunits – p180 and the B subunit (109). Professor Dornreiter's research group reported that DNA polymerase α forms two sub-populations in cells distinguishable by their phosphorylation status with the non- or hypo-phosphorylated form being the more active enzyme and present mostly at the G1/S boundary. In addition it was shown that the differentially phosphorylated forms of DNA polymerase α, associated with different cyclin/CDK complexes such that the non- or hypo- phosphorylated form of DNA polymerase α forms a complex with cyclin E-Cdk2 (and PP2A phosphatase), while the phosphorylated form preferentially forms a complex with cyclin A-Cdk2 (116). In addition, these authors showed that the phosphorylated form of DNA polymerase α was unable to bind to T antigen protein, a well-documented DNA polymerase α binding protein (45), and this inhibition could be reversed upon dephosphorylation with PP2A. The role of

each of the phosphorylation-defined sub-populations of DNA polymerase α was also examined by confocal microscopy, wherein co-localization of the phosphorylated polymerase form with active replication sites visualized by BrdU (nucleotide analog) staining was observed. Conversely, there was a lack of co-localization of the non-/hypophosphorylated form with active DNA synthesis sites. Since the non-/hypophosphorylated form appeared in the nucleoplasm but was not concentrated in regions of active DNA synthesis, it was suggested that the non-/hypophosphorylated form of DNA polymerase α might be engaged in initiation of DNA replication on the leading strand by synthesizing the first primer, while phosphorylated enzyme could be involved in primer synthesis on the lagging strand. Further investigation of this hypothesis was carried out based on co-localization and immunoprecipitation experiments with MCM2, a protein engaged in forming of pre-replication complex and then excluded from the replication fork. The different function of each DNA polymerase α, was confirmed by findings that only the non-/hypophosphorylated form appeared to co-localize and immunoprecipitate with MCM2 specifically during G1/S phase transition.

These findings regarding activity and the existence of two forms of DNA polymerase α are in accord with earlier observations of murine DNA polymerase α in which the presence of two subpopulations was also reported with regard to polyomavirus virus T antigen affinity and again distinguished the ability of these forms to initiate polyomavirus origin-dependent DNA synthesis (194).

To determine whether MDM2 might bind preferentially to one of these DNA polymerase α subpopulations, we obtained the phosphorylation status specific antibodies from Professor Dornreiter. Initial immunoprecipitation experiments were

carried out in a p53-null background on untransfected Clone 9 cells (H1299-derivative) which stably express high levels of MDM2. The results indicate that MDM2 preferentially co-immunoprecipitates with the non-/hypophosphorylated form of DNA polymerase α and this was more striking when taken together with the fact that considerably more of the phosphorylated form was immunoprecipitated (regardless of whether this was due to greater abundance or more efficient immunoprecipitation) (Figure 3.6.1). Experiments show that the non-/hypophosphorylated form is present in cells at lower levels than the phosphorylated form, and this is not surprising since it is required for initiation of replication, and after that is rapidly phosphorylated, presumably as an additional regulatory event to prevent re-replication (116). Further experiments to examine this association of MDM2 with the non-/hypophosphorylated form were performed in untransfected H1299 cells, and HEK293 cells (immortalised non-cancer derived cells that express wild-type p53) in order to test whether co-immunoprecipitation between MDM2 and non-/hypophosphorylated DNA polymerase α occurs in heterologous cells and whether MDM2 levels impact upon this. We also examined whether the presence of T antigen influenced complex formation between MDM2 and the non-/hypophosphorylated DNA polymerase in HEK293T cells. Results from these experiments indicate that the interaction between MDM2 and the non-/hypophosphorylated form of DNA polymerase α take place regardless of MDM2 expression levels, p53 status, the cells origin or the presence of T antigen.

These findings may provide a new insight regarding a role for MDM2 in events relating to initiation of DNA replication via preferential binding to a complex containing the non-/hypophosphorylated subpopulation of DNA polymerase α, since

this latter has a clear role in this process. MDM2 has been linked previously with promoting S-phase entry via interactions with the retinoblastoma protein (Rb) by inhibiting Rb function as a tumour suppressor, as well as via interactions with the E2F1 transcription factor (51,52,98,99,195). It is potentially noteworthy that E2F1 is a transcription factor that can promote expression of DNA polymerase α, hence it may be that MDM2 has an influence on DNA polymerase α activity through both indirect regulation of its expression via interaction with E2F1 and through postulated direct effects that we are only beginning to reveal in the studies presented herein. Studies performed on transgenic animal models wherein cells over-expressed MDM2, revealed that these cells went through multiple rounds of replication, either prior to cytokinesis or because they failed to undergo cytokinesis, leading to polyploidy in a p53–null background (97), and (which is more important in the present context) also in an E2F1-null background (139). These results suggest a role for MDM2 in promoting S-phase independently of p53 and E2F1. In light of these observations, we consider that our observations regarding the preferential interaction of MDM2 with the non-/hypophosphorylated form of DNA polymerase α may ultimately provide a basis for an additional mechanism by which MDM2 can impact upon S-phase promotion.

Data presented in this thesis, provide supporting evidence for an involvement of the MDM2 protein in initiation of the DNA replication process through interaction with a key S-phase initiator – non-/hypophosporylated DNA polymerase α. We therefore wanted to investigate whether we could detect any impact of MDM2 upon this process. Given limitations of time and resource, we initially examined whether the cell cycle profile would be altered upon reducing

MDM2 levels. We performed siRNA transfections targeting MDM2 in H1299 and Clone 9 (H1299) cells followed by flow cytometry analysis. The results obtained indicated that knockdown of MDM2 leads to approximately a 10% reduction in the number of cells in S phase compared to cells with unchanged MDM2 levels (Figure 3.7.3). In other studies the MDM2 knockdown in H1299 cells was observed to lead to 66% decrease in proliferation comparing to parental cells (155), supporting the idea of a role for MDM2 in p53-independent promotion of cell cycle progression and cells proliferation, which may contribute to p53-independent oncogenic potential of MDM2. However, when we examined the effect of MDM2 knock-down with siRNA and measured thymidine incorporation as an indicator of DNA synthesis we observed a smaller reduction in S-phase (c.4%) and perhaps not surprisingly in this one-off experiment, we did not detect a significant reduction in thymidine incorporation, although there was a trend towards a reduction which clearly requires further investigation (Figure 3.7.5-6).

In conclusion, since MDM2 binds preferentially to the non- /hypo-phosphorylated population of DNA polymerase α and may even have an effect on S-phase more studies are clearly warranted to shed more light on involvement of MDM2 protein in initiation of DNA replication. Such studies may subsequently lead to a better understanding of the mechanisms through which MDM2 contributes to oncogenesis. In particular, future studies need to take into account the potential involvement of other subunits of DNA polymerase α in complex formation with MDM2 in cells, as we have argued from our immunoprecipitation studies with MDM2 deletion mutants (Figure 3.2.10). It will be important to examine the potential of MDM2 to modulate the activity of the primase subunits of DNA

polymerase α given their responsibility for synthesizing RNA primers both on leading and lagging strand during DNA replication. Such an involvement of MDM2 protein in altering the primase activity of DNA polymerase α could add support to the notion of DNA replication promotion by MDM2, followed by potential increase in cells proliferation which may well contribute to MDM2 oncogenic functions.

Since DNA polymerase α, in addition to its well-documented role in the initiation of DNA replication, seems to be also engaged in cell cycle regulation (66,67), telomere maintenance (68) and epigenetic control (156,160) as described in the Introduction section of the thesis, it would also be interesting to establish whether MDM2 may influence any of these processes via interaction with DNA polymerase α. Thus this thesis begs more questions than it has answered, but hopefully has shed sufficient light on new avenues for investigation.

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